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INVENTION SPECIFIC BINDING ASSAY METHOD AND COMPOSITION, COMPOUND, AND DEVICE FOR USE THEREIN

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#29

SPECIFIC BINDING ASSAY METHOD AND COMPOSITION, COMPOUND, AND DEVICE FOR USE THEREIN

ABSTRACT OF THE DISCLOSURE

A test composition, compound, device, and method for their use in a specific binding assay which employs a substance having reactant activity, i.e., a reactant, as a labeling substance in the detection of a ligand in a liquid medium. The test composition and device comprise a conjugate formed of a specific binding substance coupled to the reactant. The reactant advantageously is an enzymatic reactant such as an enzyme substrate or coenzyme. The activity of the conjugated reactant as a constituent of a predetermined reaction is affected by reaction between the specific binding substance in the conjugate and a specific binding counterpart thereto. The presence of a ligand in a liquid medium may be determined using competitive or displacement binding or sequential saturation techniques wherein the specific binding substance in the conjugate is the ligand or a specific binding analog thereof, or using a direct binding technique wherein the specific binding substance is a specific binding partner of the ligand. The effect of the specific binding reaction on the activity of the conjugated reactant is related to the presence or amount of the ligand in the liquid medium tested.

SPECIFIC BINDING ASSAY METHOD AND COMPOSITION, COMPOUND, AND DEVICE FOR USE THEREIN

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to compositions, compounds, devices, and methods for determining the presence of a ligand in a liquid medium based on the affinity of the ligand for a specific binding partner thereof. In particular, this invention relates to compositions, devices, and methods for use in specific binding assays which do not require a separation step and which do not employ radioactive materials or modified enzymes as the labeling substance.

The desirability of a convenient, reliable, and non-hazardous means for detecting the presence of low concentrations of substances in liquids is self-evident. This is particularly true in the field of clinical chemistry where constituents of body fluids which may appear in concentrations as low as 10^{-11} molar are known to be of pathological significance. The difficulty of detecting such low concentrations is compounded in the field of clinical chemistry where sample size is usually quite limited.

Classically, substances have been detected in liquids based on a reaction scheme wherein the substance to be detected is a necessary reactant. The presence of unknown is indicated by the appearance of a reaction product or

the disappearance of a known reactant. In certain instances, such an assay method may be quantitative, based on a measurement of either the rate of appearance of product or disappearance of reactant or measurement of the aggregate amount of product produced or reactant consumed in attaining equilibrium. Each assay reaction system is necessarily either limited to use in the detection of only a small group of substances or is non-specific.

The search for assay systems which are highly specific yet adaptable to the detection of a wide range of substances has evolved the radioimmunoassay. In this system a known amount of a radiolabeled form of the substance to be detected is allowed to compete with the unknown for a limited quantity of antibody specific for the unknown. The amount of the labeled form that becomes bound to antibody varies inversely with the level of unknown present. Inherent in the radioimmunoassay technique is the need to separate the labeled form of substance to be detected which becomes bound to antibody from that which does not become so bound. While various ways of accomplishing the required separation have been developed, as exemplified in U.S. Patents Nos. 3,505,019; 3,555,143; 3,646,346; 3,720,760; and 3,793,445, all require at least one separate manipulative step, such as filtering, centrifuging, or washing, to insure efficient separation of the bound-labeled form from the unbound-labeled form. The elimination of the separation step would greatly simplify the assay and render it more useful to the clinical laboratory.

The use of radioactive materials in immunoassays has been eliminated to some degree by the use of enzyme -tagged materials in place of radiolabels. As exemplified by U.S. Patents Nos. 3,654,090 and 3,791,932, the manipulative steps necessary for carrying out the enzyme-tagged immunoassays are for the most part the same as those required in radioimmunoassays and include the cumbersome separation step. An additional disadvantage of using enzyme-tagged materials is that each enzyme used as a tag must be individually chemically modified for use in the formation of the tagged conjugate. The use of other tagging materials has been suggested, such as the use of coenzymes or viruses, *Nature 219*:186(1968) and the use of fluorescent-labels, French Patent No. 2,217,350.

2. DESCRIPTION OF THE PRIOR ART

While the radiolabeled and enzyme-tagged immunoassays may undergo future improvement in terms of expansion of the range of substances detectable thereby or of simplification of the procedure, by their nature they will always require some type of separation step. Recently, a different approach was disclosed which does not require a separation step and therefore has been referred to as a homogenous system, in contrast to a heterogenous system in which separation is essential. U.S. Patent No. 3,817,837 discloses a competitive binding assay method involving the steps of combining the liquid to be assayed with a soluble complex consisting of an enzyme as a labeling substance covalently bound to the ligand to be detected and with a soluble receptor, usually

an antibody, for the ligand; and analyzing for the effect of the liquid to be assayed on the enzymatic activity of the enzyme in the complex.

While this method has the advantage of not requiring a separation step because reaction between the enzyme-bound-ligand complex and the receptor results in inhibition of the enzymatic activity of the enzyme in the complex, the method nonetheless is severely restricted in its ability to be adapted to widely varied assay requirements. For instance, it is clearly essential that in the fabrication of the enzyme-bound-ligand complex, the substance or ligand to be detected must be coupled to the enzyme in a carefully controlled manner so that the coupling site is close to the enzymatically active site on the enzyme. This is required in order that upon reaction between the complexed ligand and the receptor, the enzymatically active site is blocked. Enzymes vary greatly in their size, ranging in molecular weight from about 10,000 to 1,000,000. Thus, for a receptor in the form of an antibody having a molecular weight of between 150,000 and 300,000 to be capable of physically blocking the active site on an average enzyme of 500,000 molecular weight or greater, the coupling site must be precisely controlled. Due to the complex chemical structure of enzymes, precise control of such chemical linkage is indeed difficult, and one would expect that even upon screening a wide variety of enzymes only a small number would be found to be of use in this homogenous assay system.

Moreover, it is critical for the purpose of obtaining quantitative test results to precisely control the ratio of the number of enzymes to the number of ligands in each

enzyme-bound-ligand complex. Here also, the complex peptide structure of enzymes makes such control difficult. It would again be expected that only a small number of enzymes would have suitable molecular structure to ensure necessary control of the ligand/enzyme ratio.

The prior art homogeneous assay method is stated to involve an enzyme amplification and thus to be highly sensitive. However, since the labeling substance, namely the enzyme, is itself the limiting factor determining the sensitivity of the prior art assay method, the versatility of the method is severely restricted. The sensitivity is clearly limited to the catalytic activity of the particular enzyme in the enzyme-bound-ligand conjugate. The versatility of the prior art method is therefore restricted not only by the coupling requirements for formation of a useful conjugate but also by the dependence of the sensitivity of the assay that employs such conjugate on the activity of the particular conjugated enzyme.

An additional disadvantage of the prior art homogeneous assay method arises in its application to the testing of biological fluids such as urine and serum. It is to be expected that significant amounts of the enzyme species comprised in the enzyme-bound-ligand conjugate may appear in the fluid sample to be tested thereby creating an uncontrollable background activity which would severely affect the accuracy of the assay method. Therefore, in order to form an assay system that is useable in testing biological fluids of humans or animals, exotic enzymes not endogenous to such

fluids must be selected for use in forming the enzyme-bound-ligand conjugate with the result that the versatility of the assay method is even further restricted.

It is therefore an object of the present invention to provide a novel test composition, compound, device, and method for detecting a ligand in a liquid which do not require a separation step and which do not employ inconvenient radioactive materials or modified enzymes as the labeling substance.

Further, it is an object of the present invention to provide a homogenous specific binding assay method and system which are more versatile and convenient than those of the prior art.

Another object of the present invention is to provide a homogenous specific binding assay method and system which employ a labeling substance which is capable of being coupled to the ligand or to a specific binding partner thereof more conveniently than can the enzyme of the prior art method.

A further object of the present inventio: is to provide a homogenous specific binding assay method and system which employ a conjugate comprising a labeling substance whose activity is more readily affected by a specific binding reaction than is the enzyme of the prior art method.

It is also an object of the present invention to provide a homogenous specific binding assay method and system which employ a conjugate comprising a labeling substance any change in the activity of which is more conveniently detectable using a wide variety of sensitive reaction systems than is any change in the activity of the enzyme in the prior art method.

It is a further object of the present invention to provide a homogenous specific assay method and system which are more readily applicable to the testing of biological fluids than those of the prior art.

SUMMARY OF THE INVENTION

The present invention provides a highly convenient, versatile, and sensitive homogenous specific binding assay method and system based on the use of, as labeling substance, a substance which exhibits given reactant activity as a constituent of a predetermined reaction, such substance being referred to herein as the reactant. The method is based, in part, on the fact that the reaction between a ligand and a specific binding partner thereof to one of which the reactant is coupled alters the activity of the reactant in the predetermined reaction. In view of this basic phenomenon, various manipulative schemes involving various test compositions and devices may be employed in performing the method of the present invention. The preferred fundamental manipulative schemes are the direct binding technique and the competitive binding technique.

In the direct binding technique, a liquid medium suspected of containing the ligand to be detected is contacted with a conjugate comprising the reactant coupled to a specific binding partner of the ligand, and thereafter any change in the activity of the reactant is assessed. In the competitive binding technique, the liquid medium is contacted with a specific binding partner of the ligand and with a conjugate comprising the reactant coupled to one or both of

the ligand or a specific binding analog thereof, and thereafter any change in the activity of the reactant is assessed. In both techniques, the activity of the reactant is determined by contacting the liquid medium with at least one reagent which forms, with the reactant, the predetermined reaction. Qualitative determination of the ligand in the liquid medium involves comparing a characteristic, usually the rate, of the resulting reaction to that of the predetermined reaction in a liquid medium devoid of the ligand, any difference therebetween being an indication of a change in activity of the reactant. Quantitative determination of the ligand in the liquid medium involves comparing a characteristic of the resulting reaction to that of the predetermined reaction in liquid media containing known amounts of the ligand.

The predetermined reaction preferably is enzyme -catalyzed. Usually, a predetermined reaction is selected which is highly sensitive to the reactant in the conjugate. Luminescent or fluorescent reaction systems are very useful in this regard. Particularly preferred are cyclic reaction systems, especially those in which the reactant is the cycled material. Of the preferred cyclic reaction systems, those which are enzyme-catalyzed are particularly advantageous. The reactant in the conjugate is usually an enzymatic reactant, such as an enzyme substrate or, as is particularly preferred, a coenzyme, and preferably has a molecular weight of less than 9000.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graphical representation of the effect of various levels of a ligand on the aggregate reaction rate in a direct binding-cycling assay technique.

Figures 2 and 3, respectively, are graphical representations of the effect of various levels of two different ligands on the aggregate reaction rate in a competitive binding-cycling assay technique.

Figures 4 and 5, respectively, are graphical representations of the effect of various levels of two different ligands on the peak light intensity produced in a competitive binding-bioluminescence assay technique.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the context of this disclosure, the following terms shall be defined as follows: ligand is the substance, or group of substances, whose presence or the amount thereof in a liquid medium is to be determined; specific binding partner of the ligand is any substance, or group of substances, which has a specific binding affinity for the ligand to the exclusion of other substances; and specific binding analog of the ligand is any substance, or group of substances, which behaves essentially the same as the ligand with respect to the binding affinity of the specific bindir, partner for the ligand.

In general, the components of the specific binding reaction, i.e., the liquid medium suspected of containing the ligand, the conjugate, and/or a specific binding partner of the ligand, may be combined in any amount, manner, and sequence, provided that the activity of the reactant in the conjugate is measurably altered when the liquid medium contains the ligand in an amount or concentration of significance to the purposes of the assay. Preferably, all of the components of the specific binding reaction are soluble in

the liquid medium, thus providing a homogenous assay system. However, a heterogenous assay system wherein the conjugate or a specific binding partner of the ligand is insoluble may be employed if desired.

Where a direct binding technique is used, the components of the specific binding reaction are the liquid medium suspected of containing the ligand and a quantity of a conjugate comprising the reactant coupled to a specific binding partner of the ligand. The activity of the conjugated reactant on contact with the liquid medium varies inversely with the extent of binding between the ligand in the liquid medium and the specific binding partner in the conjugate. Thus, as the amount of ligand in the liquid medium increases, the activity of the conjugated reactant decreases. To obtain quantitative results, the amount of the specific binding partner contacted with the liquid medium is usually in excess of that capable of binding with all of the ligand thought to be present in the liquid medium during the time that the conjugate and the liquid medium are in contact prior to completion of the assessment of any change in activity of the conjugated reactant. In practice, an amount of the specific binding partner is chosen according to the above-mentioned criterion based on an estimation of the largest amount of the ligand which is likely to be present in the liquid medium. A direct binding technique is particularly useful in detecting high molecular weight ligands which have specific binding partners that are smaller than themselves.

Where a competitive binding technique is used, the components of the specific binding reaction are the liquid

medium suspected of containing the ligand, a quantity of a conjugate comprising the reactant coupled to the ligand or a specific binding analog of the ligand, and a quantity of a specific binding partner of the ligand. The specific binding partner is contacted substantially simultaneously with both the conjugate and the liquid medium. Since any ligand in the liquid medium competes with the ligand or specific binding analog thereof in the conjugate for binding with the specific binding partner, the activity of the conjugated reactant on contact with the liquid medium varies directly with the extent of binding between the ligand in the liquid medium and the specific binding partner. Thus, as the amount of the ligand in the liquid medium increases, the activity of the conjugated reactant increases. To obtain quantitative results, the amount of the specific binding partner contacted with the conjugate and the liquid medium is usually less than that capable of binding with all of the ligand thought to be present in the liquid medium and all of the ligand or ligand analog in conjugated form in the time that the specific binding partner, the conjugate, .nd the liquid medium are in contact prior to completion of the assessment of any change in activity of the conjugated reactant. In practice, an amount of the specific binding partner is chosen according to the above-mentioned criterion based on an estimation of the largest amount of the ligand which is likely to be present in the liquid medium. Usually, the amount of the ligand or ligand analog in conjugated form which is contacted with the liquid medium does not exceed the smallest amount of the ligand to be tested for in the

liquid medium. A competitive binding technique is particularly useful in detecting ligands which have specific binding partners that are larger than themselves.

A variation of the competitive binding technique is the displacement binding technique wherein the conjugate is contacted first with the specific binding partner of the ligand and thereafter with the liquid medium. Competition for the specific binding partner then occurs. In such a method, the amount of the conjugate contacted with the specific binding partner is usually that which comprises the ligand or analog thereof in excess of that capable of binding with the amount of the specific binding partner present during the time that the conjugate and the specific binding partner are in contact prior to contact with the liquid medium suspected of containing the ligand. This order of contact may be accomplished in either of two convenient ways. In one method, the conjugate is contacted with the specific binding partner in a liquid environment prior to contact with the liquid medium suspected of containing the ligand. In the second method, the liquid medium suspected of containing the ligand is contacted with a complex comprising the conjugate and the specific binding partner, the specific binding substance in the conjugate and the specific binding partner being reversibly bound to each other. The amount of the conjugate that becomes bound to the specific binding partner in the first method, as well as the amount thereof which is in complexed form in the second method, is

usually in excess of that capable of being displaced by all of the ligand in the liquid medium in the time that the specific binding partner, or complex, and the medium are in contact prior to the completion of the assessment of any change in the activity of the conjugated reactant.

Another variation of the competitive binding technique is the sequential saturation technique wherein the components of the specific binding reaction are the same as those used in the competitive binding technique, but the order of addition or combination of the components and the relative amounts thereof used are different. Following a sequential saturation technique, the specific binding partner of the ligand is contacted with the liquid medium suspected of containing the ligand for a period of time prior to the contact of said liquid medium with the conjugate. The amount of the specific binding partner contacted with the liquid medium is usually in excess of that capable of binding with all of the ligand thought to be present in the liquid medium in the time that the specific binding partner and the liquid medium are in contact prior to the time that the liquid medium is contacted with the conjugate. Further, the amount of the ligand or ligand analog in conjugated form is usually in excess of that capable of binding with the remaining unbound amount of the specific binding partner during the time that the liquid medium and the conjugate are in contact prior to the completion of the assessment of any change in activity of the conjugated

reactant. In practice, the amounts of the specific binding partner and of the ligand or ligand analog in conjugated form are chosen according to the above-mentioned criterion by estimating the largest amount of the ligand likely to be present in the liquid medium.

It is contemplated that manipulative schemes involving other orders of addition and other relative amounts of the specific binding reaction components may be devised for carrying out a homogenous specific binding assay without departing from the inventive concept embodied herein.

The step of assessing any change in activity of the conjugated reactant as a constituent of the predetermined reaction is conveniently accomplished by contacting the specific binding reaction mixture with at least one substance which forms with the conjugated reactant, the predetermined reaction, and determining the effect of the specific binding reaction on a characteristic of such reaction. The predetermined reaction may comprise a single chemical transformation or a plurality or series of chemical transformations. Unless otherwise specified, the term "reaction system" as used herein refers to the whole or a portion of the predetermined reaction.

Where an enzyme-catalyzed reaction system is used, it includes, in addition to the conjugated reactant, at least one enzyme and may include one or more enzymatic reactants such as substrates and coenzymes. Such enzyme-catalyzed reaction system may comprise a single simple enzymatic reaction or a complex series of enzymatic and non-enzymatic reactions. For instance, the enzyme-catalyzed reaction system may consist of

a single enzyme-catalyzed degradation or dissociation reaction. In such a system, the conjugated reactant is the enzyme substrate which undergoes degradation or dissociation, and the only component of the reaction system necessary to be contacted with the specific binding reaction mixture is an enzyme which catalyzes the degradation or dissociation reaction. A more complex enzyme-catalyzed reaction system may consist of a single enzymatic reaction involving two or more reactants or may consist of a series of reactions involving several reactants, at least one of which reactions is enzyme-catalyzed. In such a system, the conjugated reactant would be one of the enzymatic reactants in the enzyme-catalyzed reaction and the specific binding reaction mixture would be contacted with the appropriate enzyme and reactant constituents, other than that in the conjugate, which are necessary to provide the selected enzyme-catalyzed reaction system.

It is further contemplated that the enzyme-catalyzed reaction system may comprise a biochemical system as complex as the metabolic system of a biological cell such as a microorganism. For example, a nutrient substance essential to the growth of a particular microorganism may be selected as the reactant in the conjugate. Any change in the activity of the reactant would cause a change in a growth characteristic of the microorganism when such microorganism would be placed in an environment wherein the only source of the reactant nutrient substance is the conjugate. Thus, for example, a change in the rate of microorganism growth when contacted with the specific binding reaction mixture would indicate the presence of the ligand therein.

The appropriate reaction constituents which form, together with the reactant in the conjugate, the predetermined reaction may be contacted with the specific binding reaction mixture singularly or in any combination either prior to, simultaneous with, or subsequent to initiation of the specific binding reaction. After initiation of the specific binding reaction, the reaction mixture, which may include any or all of the necessary components for the predetermined reaction is usually incubated for a predetermined period of time before assessing any change in the activity of the reactant in the conjugate. After the incubation period, any components which are necessary for the predetermined reaction and which are not already present in sufficient quantities in the reaction mixture are added thereto, and any effect on the predetermined reaction is assessed as an indication of the presence or amount of the ligand in the liquid medium.

In the situation where the ligand is absent from the liquid medium, or is present in an insignificantly small amount, the predetermined reaction exhibits a relatively constant character. When the ligand is present in the liquid medium, at least one characteristic or property of the predetermined reaction is altered. Generally, the activity of the conjugated reactant is defined as the extent or rate at which the reactant is capable of participating in the predetermined reaction. Thus, the character of the predetermined reaction is altered by the presence of the ligand in the liquid medium, usually with respect to either the aggregate reaction rate thereof or the equilibrium quantity

of one or more reaction products produced thereby. In the usual case, the ability of the conjugated reactant to participate in the predetermined reaction is decreased upon reaction between the specific binding substance to which it is conjugated and a specific binding counterpart of such specific binding substance, that is, the conjugate in its free state is more active in the predetermined reaction than in its bound state. The relative amounts of free and bound conjugate present after the incubation of the specific binding reaction are a function of the amount of ligand in the liquid medium and are determinative of the effect on the predetermined reaction.

When the change in the aggregate reaction rate of the predetermined reaction is the characteristic used to determine the presence of the ligand, as is preferred, such rate is usually determined by measuring the rate of disappearance of a reactant or the rate of appearance of a reaction product. Such measurement can be accomplished by a wide variety of methods including the conventional chromatographic, gravimetric, potentiometric, spectrophotometric, fluorometric, turbidimetric, and volumetric analysis techniques. Since the present method is primarily designed for the detection of low concentrations of ligands, highly sensitive reaction systems have been developed for use in conjunction with the novel specific binding reaction system.

One preferred form of the predetermined reaction includes a luminescent reaction system, preferably enzyme-catalyzed, such as a reaction exhibiting the phenomenon of bioluminescence or chemiluminescence. The reactant in the conjugate

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TABLE A

	Luminescent Reaction System	Conjugated Reactant
A.	ATP + reduced luciferin $\xrightarrow[\text{(fire fly)}]{\text{luciferase}}$ $h\nu$ + AMP + oxidized luciferin	ATP or reduced luciferin
B.	$FMNH_2$ + long-chain aldehyde + O_2 $\xrightarrow[(\textit{P. fisheri})]{\text{luciferase}}$ $h\nu$ + FMN + long-chain acid + H_2O	$FMNH_2$ or long-chain aldehyde
C.	1) NADH + FMN + $H^{\oplus}$ $\xrightarrow{\text{NADH dehydrogenase}}$ NAD + $FMNH_2$ 2) $FMNH_2$ + long-chain aldehyde + O_2 $\xrightarrow[(\textit{P. fisheri})]{\text{luciferase}}$ $h\nu$ + FMN + long-chain acid + H_2O	NADH or FMN
D.	1) 3',5'-adenosine diphosphate + reduced luciferin sulfate $\xrightarrow{\text{sulfate transferase}}$ adenosine-3'-phosphate-5'-phosphosulfate + reduced luciferin 2) reduced luciferin + O_2 $\longrightarrow$ $h\nu$ + oxidized luciferin	3'5'-adenosine diphosphate or reduced luciferin

TABLE A

	Luminescent Reaction System	Conjugated Reactant
E.	reduced luminol + $H_2O_2 \xrightarrow{\text{peroxidase}^*}$ $h\nu$ + oxidized luminol + H_2O	reduced luminol
F.	reduced pyrogallol + $H_2O_2 \xrightarrow{\text{peroxidase}^*}$ $h\nu$ + oxidized pyrogallol + H_2O	reduced pyrogallol
G.	reduced luminol + $O_2 \xrightarrow{\text{oxygenase}}$ $h\nu$ + oxidized luminol	reduced luminol
H.	reduced pyrogallol + $O_2 \xrightarrow{\text{oxygenase}}$ $h\nu$ + oxidized pyrogallol	reduced pyrogallol

*or catalase

Further details and discussion concerning luminescent reaction systems which may be used in the present method may be found in the following references:

J. Biol. Chem. *236*:48(1961).

J. Amer. Chem. Soc. *89*:3944(1967).

Cornier et al., *Bioluminescence in Progress*, ed. Johnson et al., Princeton University Press (New Jersey, 1966) pp. 363-84.

Kries, P. *Purification and Properties of Renilla Luciferase*, doctoral thesis University of Georgia (1967).

Am. J. Physiol. *41*:454(1916).

Biol. Bull. *51*:89(1926).

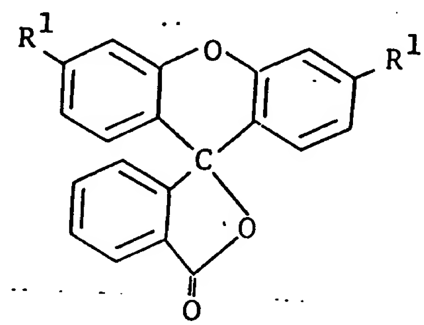
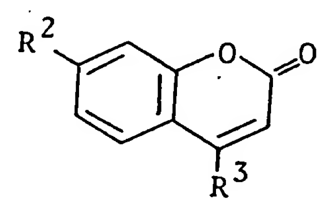
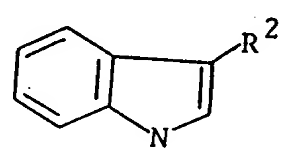
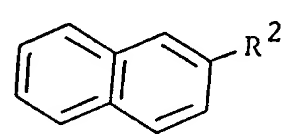
J. Biol. Chem. *243*:4714(1968).

Another type of preferred, sensitive, predetermined reaction involves the phenomenon of fluorescence and is enzyme-catalyzed. In such a reaction system the reactant in the conjugate is a substrate in an enzymatic reaction which produces a product which has fluorescent properties that differ from those of the conjugated substrate. Any change in the activity of the conjugated enzymatic reactant resulting from the specific binding reaction causes a change in the fluorescent properties of the reaction mixture. A general reaction scheme for such an enzyme-catalyzed reaction system is as follows:

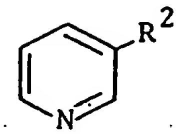
$$\begin{array}{l}\text{enzymatic}\\ \text{reactant}\\ \text{(substrate)}\end{array}\text{-X-Z} \xrightarrow{\text{(enzyme)}} \text{product}$$

wherein X is an enzyme-cleavable bond or linking group, such as an ester or amido group, and Z is a specific binding sub-

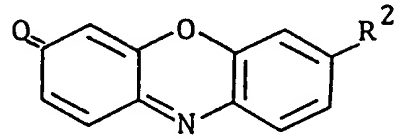
stance which, depending upon the specific binding reaction technique used, is the ligand, a specific binding analog of the ligand, or a specific binding partner of the ligand. Specific conjugates which may be used in this type of reaction system are various enzyme-cleavable derivatives of fluorescein, umbelliferone, 3-indole, ß-naphthol, 3-pyridol, resorufin, rhodamine B, and so forth. Examples of possible structural formulas of such derivatives are as follows:

Derivative	Formula
fluorescein	
umbelliferone	
3-indole	
ß-naphthol	

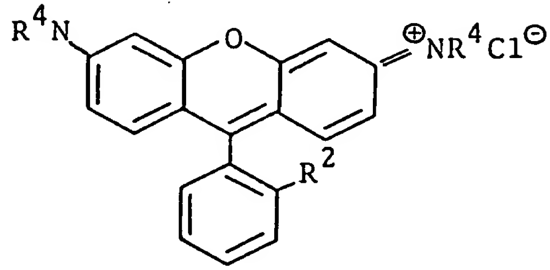
3-pyridol



resorufin



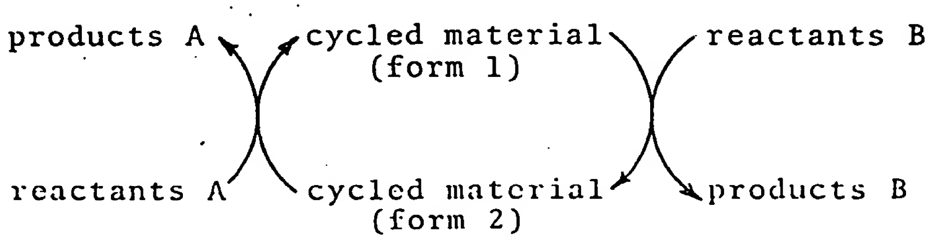
rhodamine B



wherein R^1 is -OH or -X-Z (as defined above in this paragraph), R^2 is -X-Z, R^3 is -H or $-CH_3$, and R^4 is $-(CH_2CH_3)_2$.

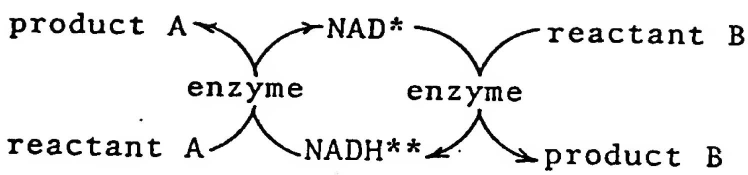
A reaction system which is particularly preferably for use in conjunction with the novel specific binding reaction of the present invention is a cyclic or cycling reaction system. Such a reaction system is one in which a product of a first reaction is a reactant in a second reaction, which second reaction has as one of its products a substance that is also a reactant in the first reaction.

The following diagram illustrates a model of a cyclic reaction system:



In the above model cyclic reaction system, a small amount of cycled material, if provided with sufficient amounts of reactants A and B, will generate large amounts of products A and B. Since the rate and amount of product produced by the reactions constituting the cyclic reaction system is highly sensitive to the amount of cycled material present, it is most preferred to use the cycled material as the reactant in the conjugate of the present invention. Examples of cycling reaction systems contemplated for use in conjunction with the novel specific binding reaction system of the present invention are given in Tables B,C, and D.

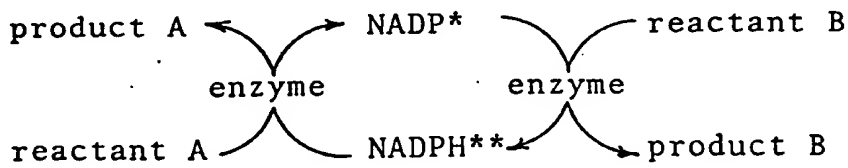
TABLE B



reaction	reactant A or product B	enzyme	reactant B or product A
1	lactaldehyde	alcohol dehydrogenase	propanediol
2	α-ketoglutarate + NH_3	glutamic dehydrogenase	glutamate
3	oxaloacetate	malic dehydrogenase	malate
4	acetaldehyde	alcohol dehydrogenase	ethanol
5	α-ketoglutarate + CO_2	isocitric dehydrogenase	isocitrate
6	dehydroxyacetone phosphate	α-glycerol phosphate dehydrogenase	L-α-glycerol phosphate
7	pyruvate	lactic dehydrogenase	lactate
8	1,3-dephosphoglycerate	glyceraldehyde-3-phosphate dehydrogenase	glyceraldehyde-3-phosphate + phosphate

* nicotinamide adenine dinucleotide
** reduced NAD

TABLE C



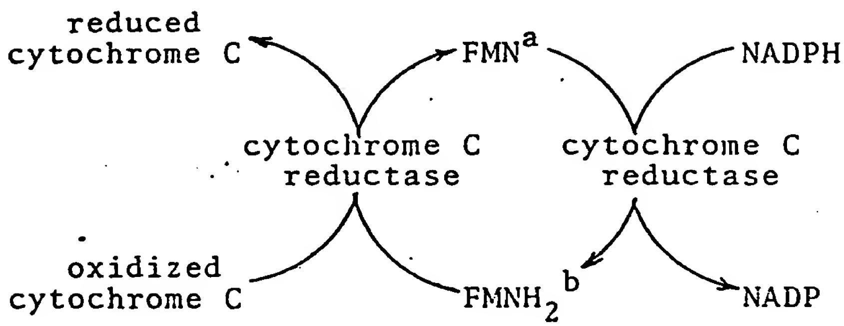
reaction	reactant A or product B	enzyme	reactant B or product A
1	6-phospho-gluconate	glucose-6-phosphate dehydrogenase	glucose-6-phosphate
2	oxidized glutathione	glutathione reductase	reduced glutathione
3	ρ-benzoquinone	quinone reductase	hydroquinone
4	nitrate	nitrate reductase	nitrite
5	α-ketoglutarate + NH_3	glutamic dehydrogenase	glutamate

* nicotinamide adenine dinucleotide phosphate
** reduced NADP

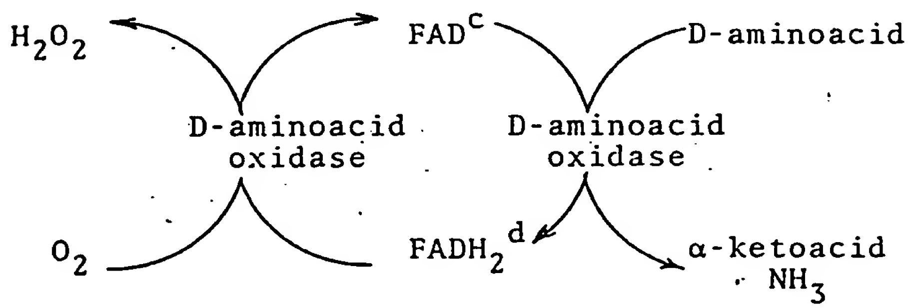
It should be noted that the cyclic reaction systems illustrated in Tables B and C comprise the combination of any one of the reactions listed in the respective tables with any other reaction listed therein. For example, reaction 1 in Table B may be paired with any one of reactions 2-9 to form a useful cyclic reaction system. Thus, Tables B and C represent respectively 56 and 20 possible cyclic reaction systems for use in the present invention.

In addition to the cyclic reaction systems represented in Tables B and C, it is contemplated that one of the reactions in the cyclic reaction system may involve the enzymatic or non-enzymatic conversion of a spectrophotometric indicator, preferably colorimetric. In such a system, any change in the reaction or cycling rate would be reflected in a change in the spectrophotometric properties of the indicator. Using the preferred colorimetric indicators such change would be a color change. An example of a cyclic reaction system involving a conversion of an indicator is the system produced by combining one of the reactant B - product B reactions from Table B with a reaction comprising an oxidation-reduction indicator and an electron transfer agent. As electron transfer agent, phenazinemethosulfate may be used. Useful indicators include the oxidized forms of nitrotetrazolium, thiazoyl blue, and dichlorophenolindophenol.

TABLE D



[a]flavin mononucleotide
[b]reduced FMN



[c]flavin adenine dinucleotide
[d]reduced FAD

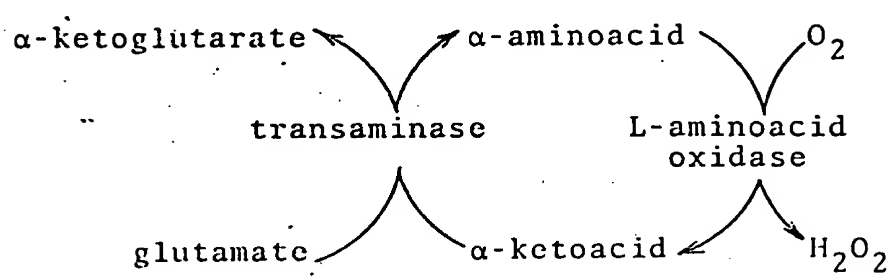
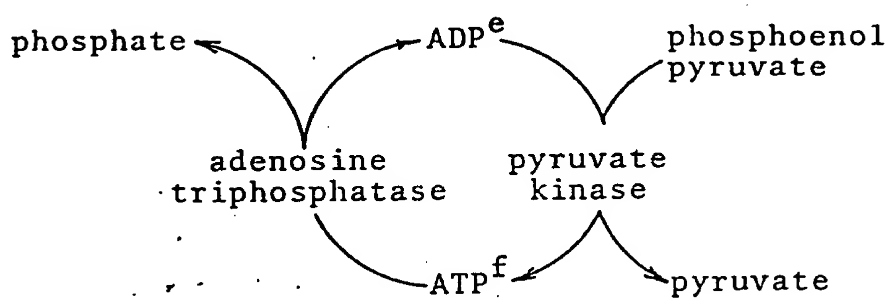
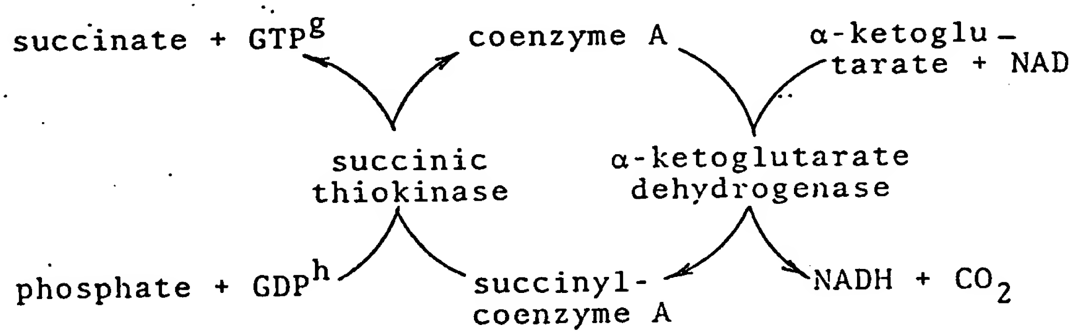


TABLE D



[e]adenosine diphosphate
[f]adenosine triphosphate



[g]guanosine triphosphate
[h]guanosine diphosphate

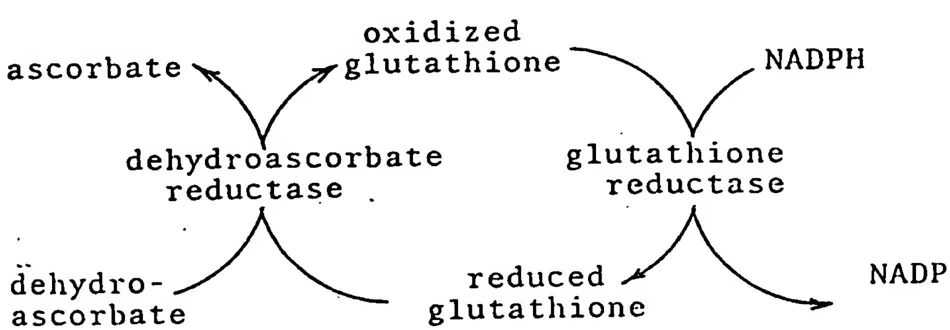
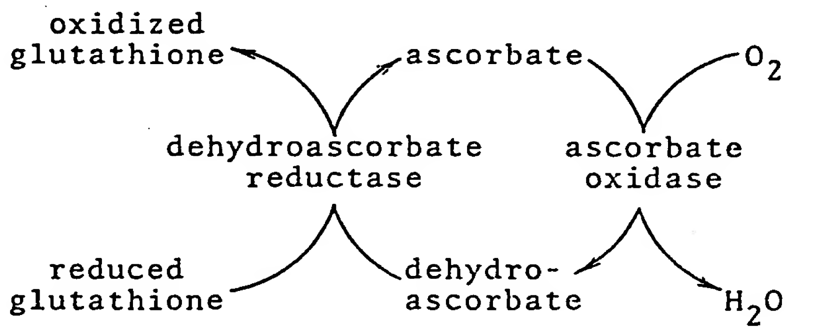
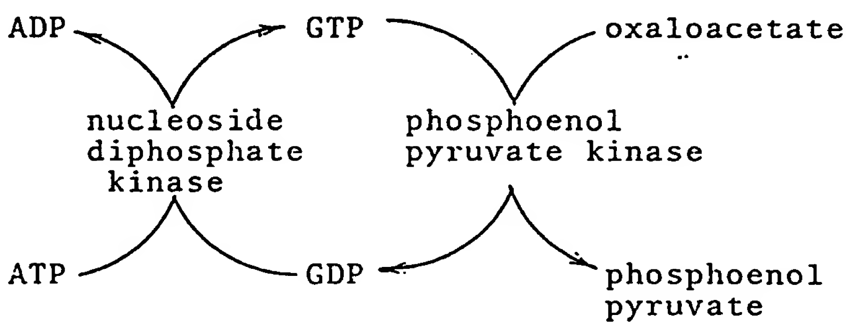


TABLE D


oxidized glutathione
ascorbate
O_2
dehydroascorbate reductase
ascorbate oxidase
reduced glutathione
dehydro-ascorbate
H_2O


ADP
GTP
oxaloacetate
nucleoside diphosphate kinase
phosphoenol pyruvate kinase
ATP
GDP
phosphoenol pyruvate

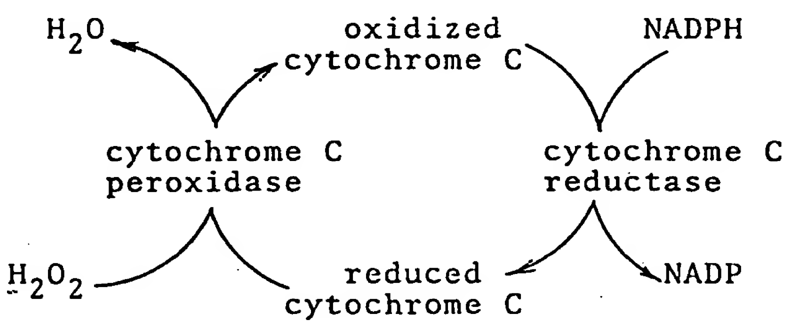
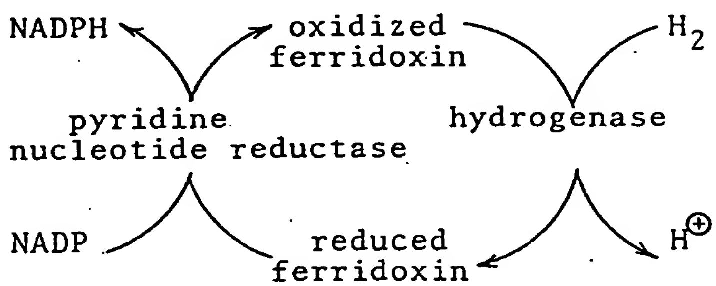

H_2O
oxidized cytochrome C
NADPH
cytochrome C peroxidase
cytochrome C reductase
H_2O_2
reduced cytochrome C
NADP

TABLE D



In forming any of the cyclic reaction systems illustrated in Tables B,C, and D, where a component in the reaction system is in an ionic form, it may of course be added in a salt or acid form which is ionizable upon contacting the liquid medium. A water soluble salt or acid of such component is usually preferred.

It is also contemplated that an exponential cyclic reaction system may be included in the predetermined reaction system. An example of an exponential cyclic reaction system is as follows:

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} 2\ \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

Such a cyclic reaction is autocatalytic in the sense that during each cycle the amount of cycled material is doubled. The cycling rate therefore increases exponentially with time and affords a high degree of sensitivity. Further details and discussion relating to such cyclic reactions may be found in *J. Biol. Chem. 247*;3558-70(1972).

Where a cyclic reaction system is used as a means of assessing any change in activity of the conjugated reactant, the rate of disappearance of a reactant or rate of appearance of a reaction product can be determined by conventional techniques or by using one or more additional cycling systems followed by a conventional determination of the aggregate reaction rate.

The use of a cyclic reaction system in conjunction with the specific binding reaction system provides a high degree of assay versatility as well as sensitivity. A single reactant-specific binding substance conjugate may be used with a multiplicity of reactions to form cyclic systems which have sensitivities varying over a wide range and which provide a wide variety of responses detectable by the senses or artificial means. Such versatility is lacking in the homogenous enzymatic assay system of the prior art.

While unnecessary in the preferred embodiment of the present invention, it may be desirable to employ a heterogenous assay technique even where the presence of the ligand in the liquid medium affects the activity of the conjugated reactant. Such a situation may present itself where a heterogenous system offers particular convenience. Certain heterogenous systems have the ability to increase the effective concentration of the ligand in the assay system, thus increasing sensitivity. An example of such a heterogenous system is that which employs a column device containing an insoluble matrix comprising either the conjugate of the present invention or a specific binding partner of the ligand,

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In general, it is preferred that the conjugate comprise the reactant coupled to the smaller of the ligand and its selected specific binding partner. It is preferred to use a direct binding technique to detect the ligand where the molecular weight of the selected specific binding partner is about one-tenth that of the ligand or less. Thus, where the ligand to be detected is an antibody or a specific binding receptor, it is preferred to follow a direct binding technique wherein the conjugate comprises an enzymatic reactant partner is ten or more times larger than that of the ligand to be detected, as when an antigen, hapten, hormone, vitamin, metabolite or pharmacological agent is to be detected, it is particularly advantageous to employ a competive binding or sequential saturation technique in which the conjugate comprises the reactant coupled to the smaller ligand.

In the conjugate of the present invention, the reactant is coupled or bound to a specific binding substance, which is the ligand, a specific binding analog of the ligand, or a specific binding partner of the ligand depending upon the assay scheme selected, such that a measurable amount of activity of the reactant is retained. The bond between the reactant and the specific binding substance is usually substantially irreversible under the conditions of the assay such as where the predetermined reaction in which the reactant has activity is not designed to chemically destroy such

bond as in the above-mentioned luminescent and cyclic reaction systems. However, in certain instances such bond is by design destroyed or otherwise affected by the selected predetermined reaction as a means for assessing the change in reactant activity. Such a case is the enzymatic fluorescent substrate reaction systems referred to previously herein.

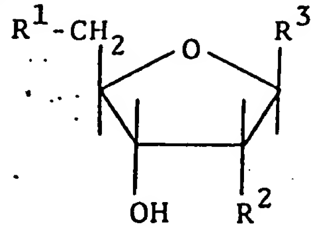
The reactant may be directly coupled to the specific binding substance so that the molecular weight of the conjugate is less than or equal to the aggregate molecular weight of the reactant and the specific binding substance. Usually, however, the reactant and the specific binding substance are linked by a bridge group comprising between 1 and 50, and preferably between 1 and 10, carbon atoms or heteroatoms such as nitrogen, oxygen, sulfur, phosphorus and so forth. Examples of a bridge group comprising a single atom would be a methylene group (one carbon atom) and an amino group (one heteroatom). The bridge group usually has a molecular weight not exceeding 1000 and preferably less than 200. The bridge group comprises a chain of carbon atoms or heteroatoms, or a combination of both, and is joined to the reactant and the specific binding substance, or active derivative thereof, by a connecting group usually in the form of an ester, amido, ether, thioester, thioether, acetal, methylene, or amino group.

The reactant in the conjugate of the present invention may be any substance which has given (i.e. fixed or known) reactant activity as a constituent of a predetermined reaction. Preferably, the reactant is an enzymatic reactant such as an enzyme substrate or, as is particularly preferred, a coenzyme. An enzyme substrate is a compound capable of undergoing a chemical transformation that is catalyzed by an enzyme. Where a substrate is employed as the conjugated reactant, the preferred molecular weight thereof is less than 9000 and preferably less than 5000. Substrates of such size, because of their lack of molecular complexity, are most convenient for use in the fabrication of the conjugate. Moreover, the activity of such substrates when coupled to a specific binding substance is readily affected by reaction of the conjugate with a specific binding counterpart of such specific binding substance. Examples of enzyme substrates which are contemplated for use in the present invention include the enzyme-cleavable fluorescent substrates referred to previously such as fluorescein and umbelliferone derivatives; pH indicators; and spectrophotometric indicator dyes, particularly chromogenic types.

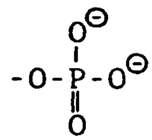
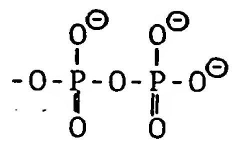
For the above reasons and for reasons of versatility and adaptability, coenzymes are especially preferred for use as the reactant in the conjugate. A coenzyme is a non-protein molecule which migrates from one enzyme protein to another in facilitating the efficient performance of the catalytic function of the enzyme. All known coenzymes have a molecular weight of less than 9000, the preferred coenzymes having a molecular weight of less than about 5000.

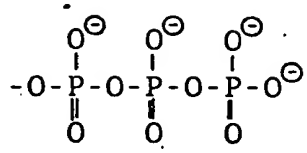
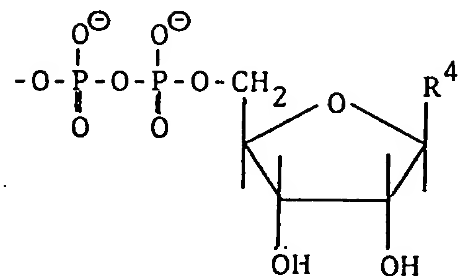
Useful coenzymes include the nucleotide coenzymes, particularly those comprising adenine groups, such as the adenosine phosphates (i.e. the mono-, di-, and tri-phosphate forms), nicotinamide adenine dinucleotide and its reduced forms, and nicotinamide adenine dinucleotide phosphate and its reduced forms. Other useful coenzymes include the guanosine phosphates, flavin mononucleotide and its reduced forms, flavin adenine dinucleotide and its reduced forms, coenzyme A and its thioesters including succinyl-coenzyme A, 3',5' adenosine diphosphate, and adenosine-3'-phosphate-5'-phosphosulfate.

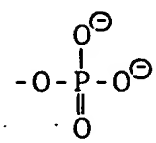
Useful coenzyme-active conjugates comprise nucleotide coenzymes having an adenine group to which the specific binding substance, i.e., a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand, is coupled through a direct bond or a bridge group as referred to hereinbefore. Such coenzyme-active conjugates which comprise an adenosine phosphate, nicotinamide adenine dinucleotide or its reduced form, or nicotinamide adenine dinucleotide phosphate or its reduced form, have the following general formula:



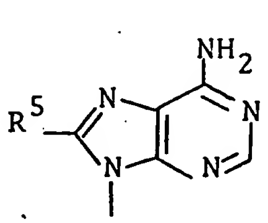
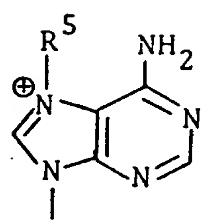
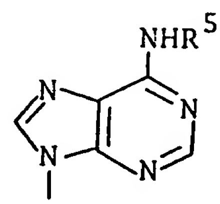
wherein R^1 is

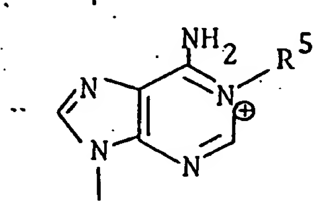
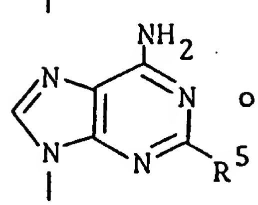
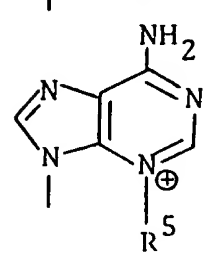
 ,  ,

 , or  ;

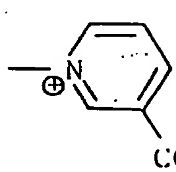
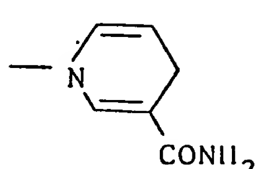
wherein R^2 is -OH or ;

wherein R^3 is

 ,  ,  ,

 ,  or  ;

wherein R^4 is

 or  ;

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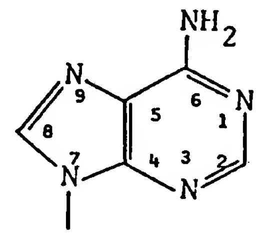
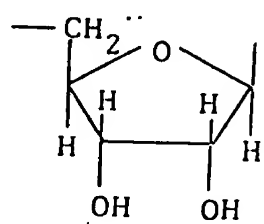
Synthesis of such compounds may be accomplished in a variety of ways. It is contemplated that the synthesis routes which are schematically illustrated below are advantageously followed in the preparation of the useful compounds. In the illustrated syntheses, the positions on the adenine ring structure are referred to according to the following:

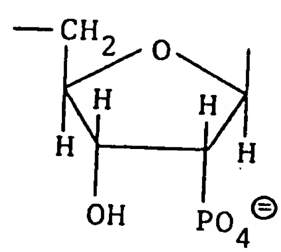


Also, the following abbreviations are used;

Rib refers to the ribose moeity, i.e.,



Rib' refers to the phosphated ribose moeity, i.e.,



Ph refers to a phosphate group;

AP derivatives refers to derivatives of adenosine -5'-phosphate, i.e., the mono- (AMP), di- (ADP), or tri- (ATP) phosphate form;

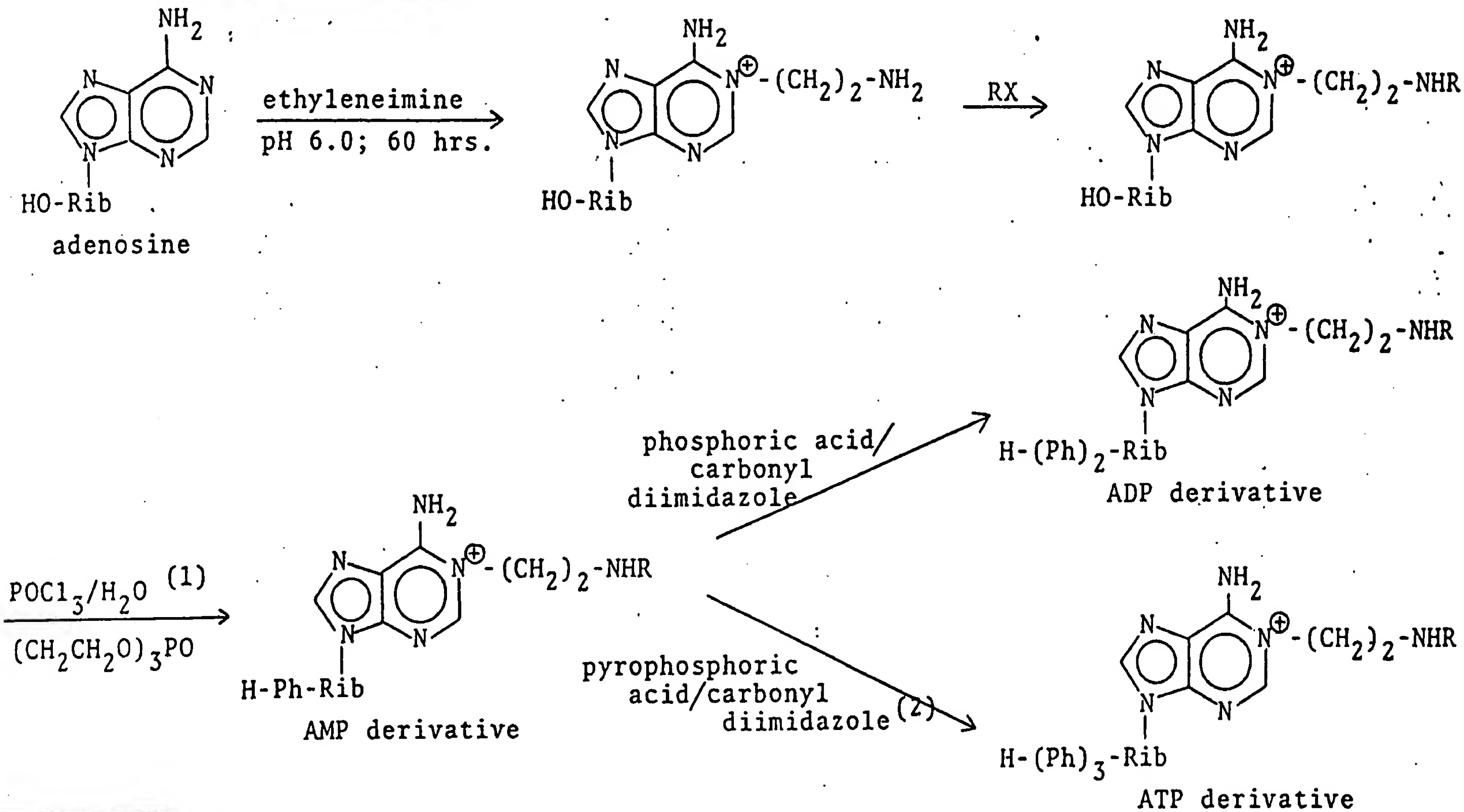
NAD derivative refers to a derivative of either nicotinamide adenine dinucleotide or a reduced form thereof;

NADP derivative refers to a derivative of either nicotinamide adenine dinucleotide phosphate or a reduced form thereof;

R refers to the specific binding substance; and

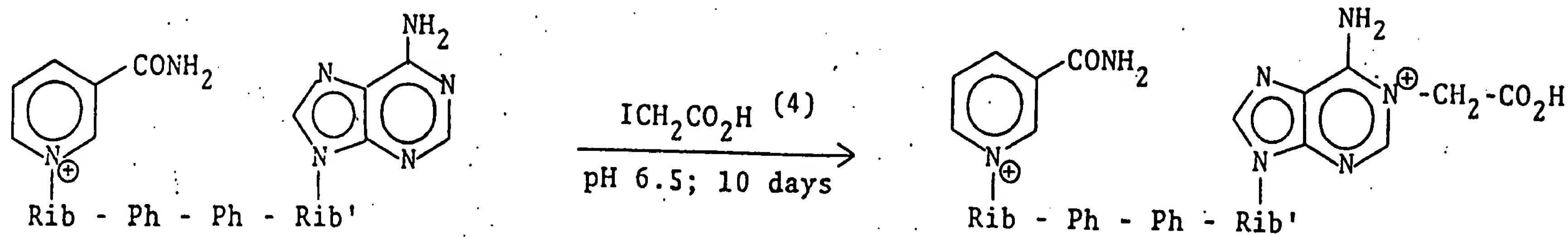
X refers to a leaving group, usually a halogen.

1-position derivatives of AP

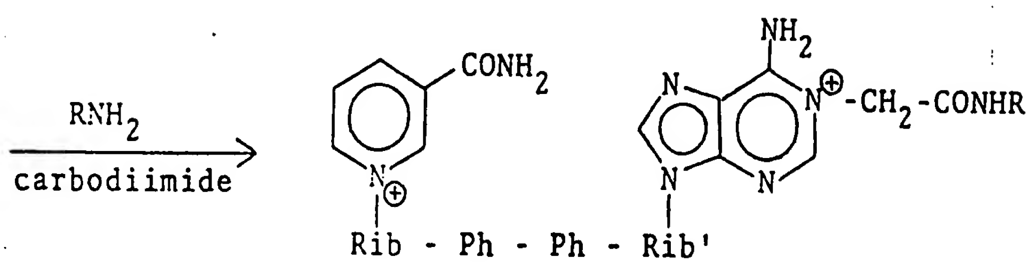


(1) Guilford, H., et al., *Chemica Scripta 2*:165 (1972).
(2) Trayer, I. P., et al., *Biochem. J. 139*:609 (1974).

<u>1-position derivative of NADP</u>



NADP



NADP derivative

(4) Lowe, C.R. and Mosbach, K., *Eur. J. Biochem.* 49:511 (1974).

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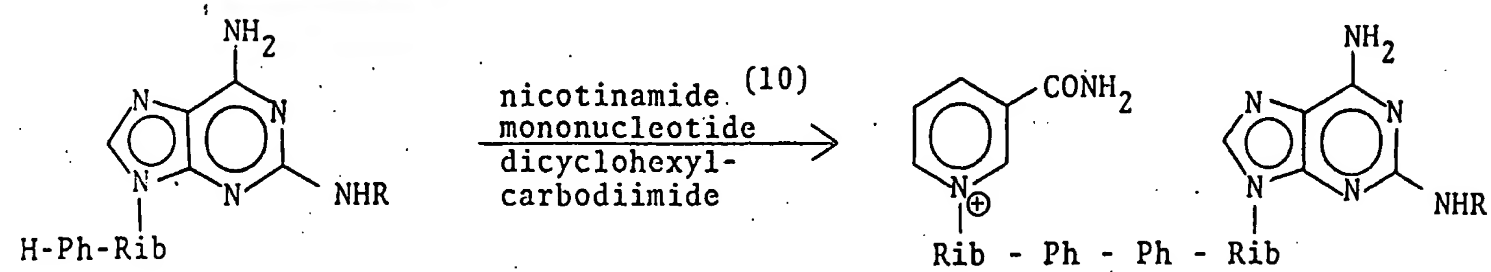
2-position derivative of NAD

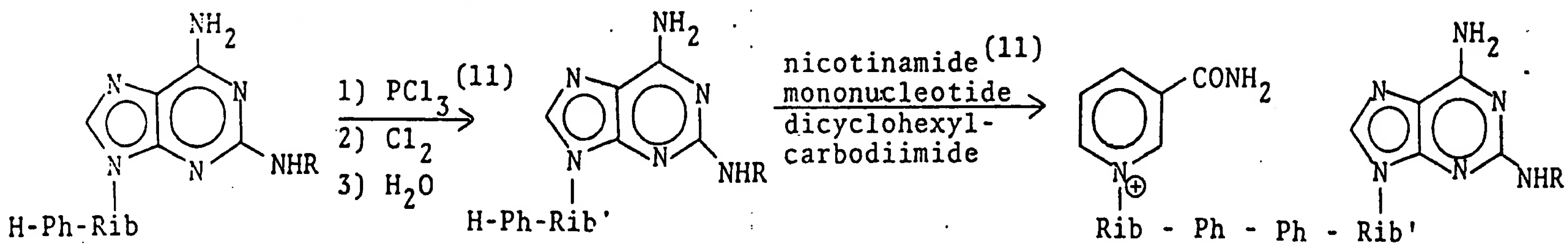


AMP derivative

NAD derivative

(10) Hughes, N.A., et al., *J. Chem. Soc.*, 3733 (1957).

2-position derivative of NADP

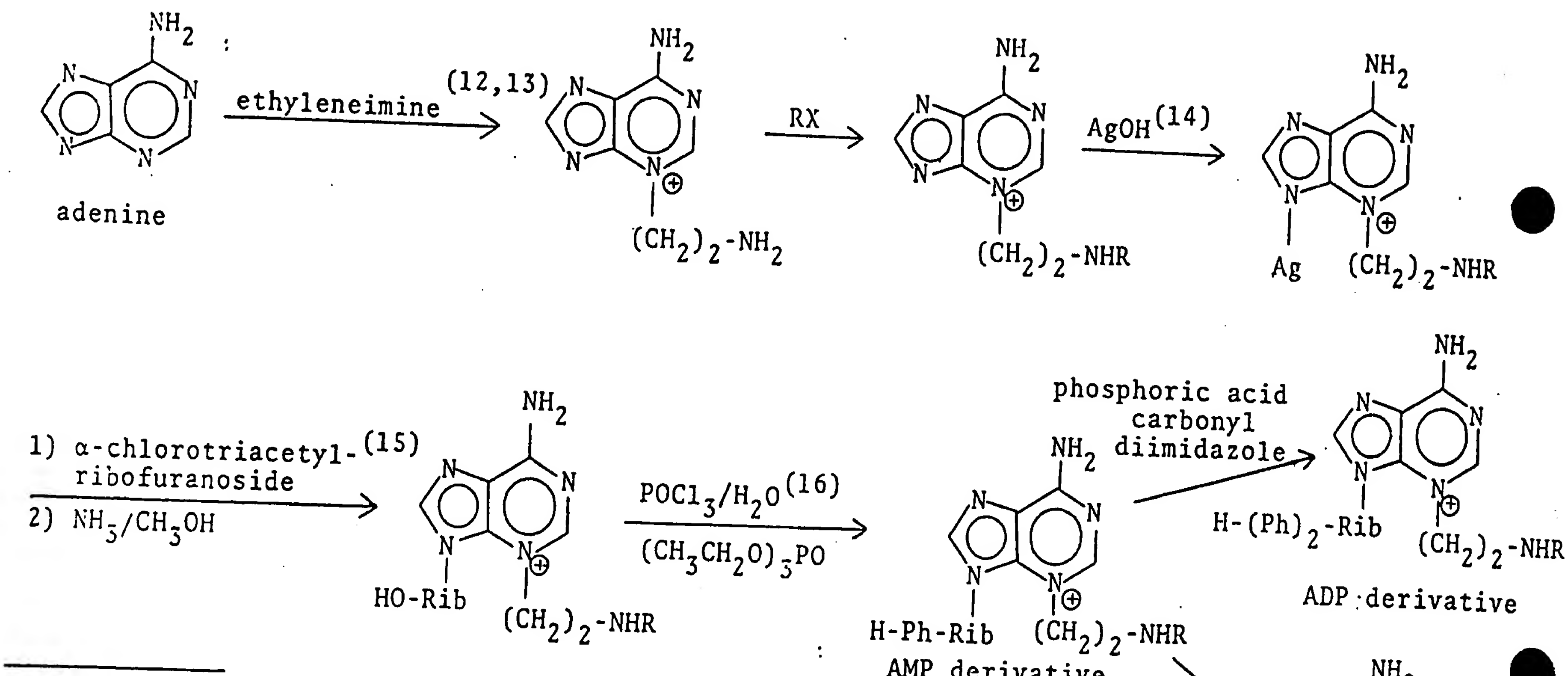


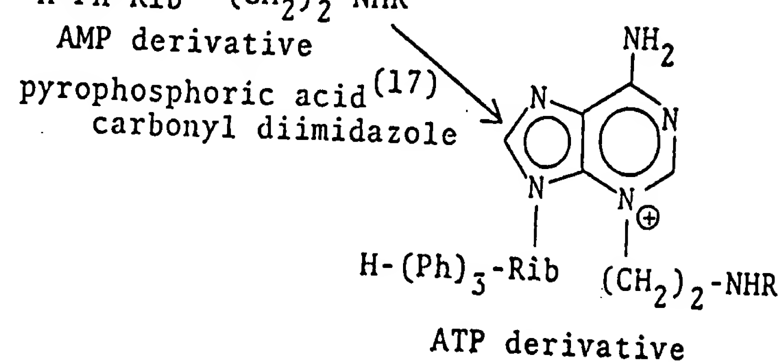
AMP derivative

NADP derivative

(11) Hughes, N.A., et al., supra.

3-position derivatives of AP





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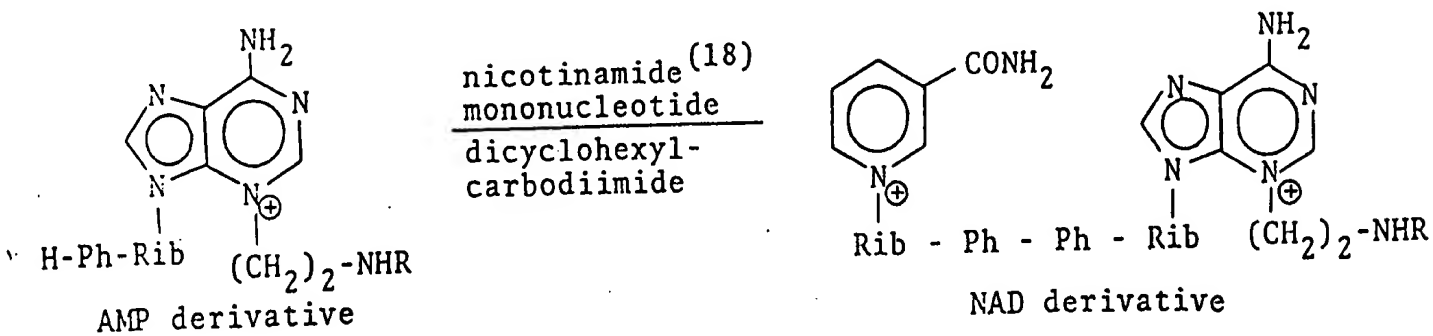
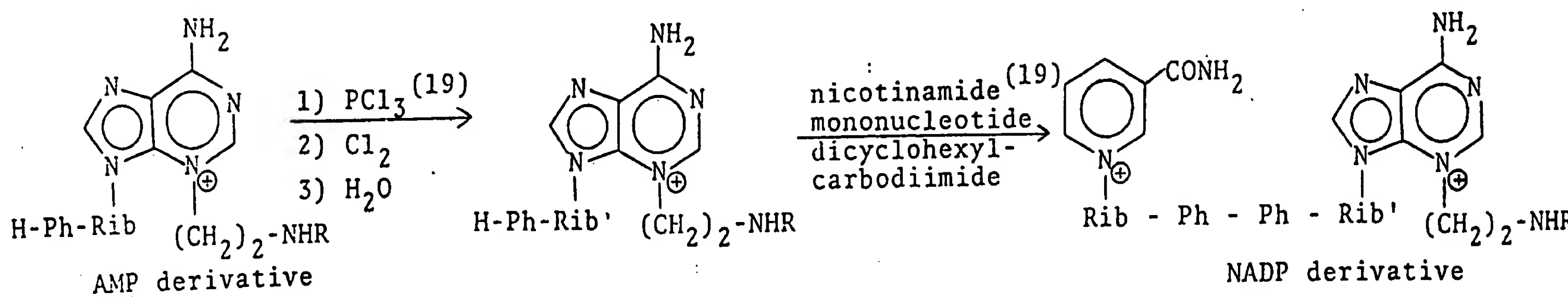
(12) Lister, J.H., in *Advances in Heterocyclic Chemistry*, ed. Katritzky et al., Academic Press (New York, 1966), p. 33.
(13) Leonard, N.J., and Fujii, T.J., *J. Amer. Chem. Soc.* *85*:3719 (1963).
(14) Fischer, E., supra.
(15) Davoll et al., supra.
(16) Guilford, H., et al., supra.
(17) Trayer, I.P., et al., supra.

3-position derivative of NAD



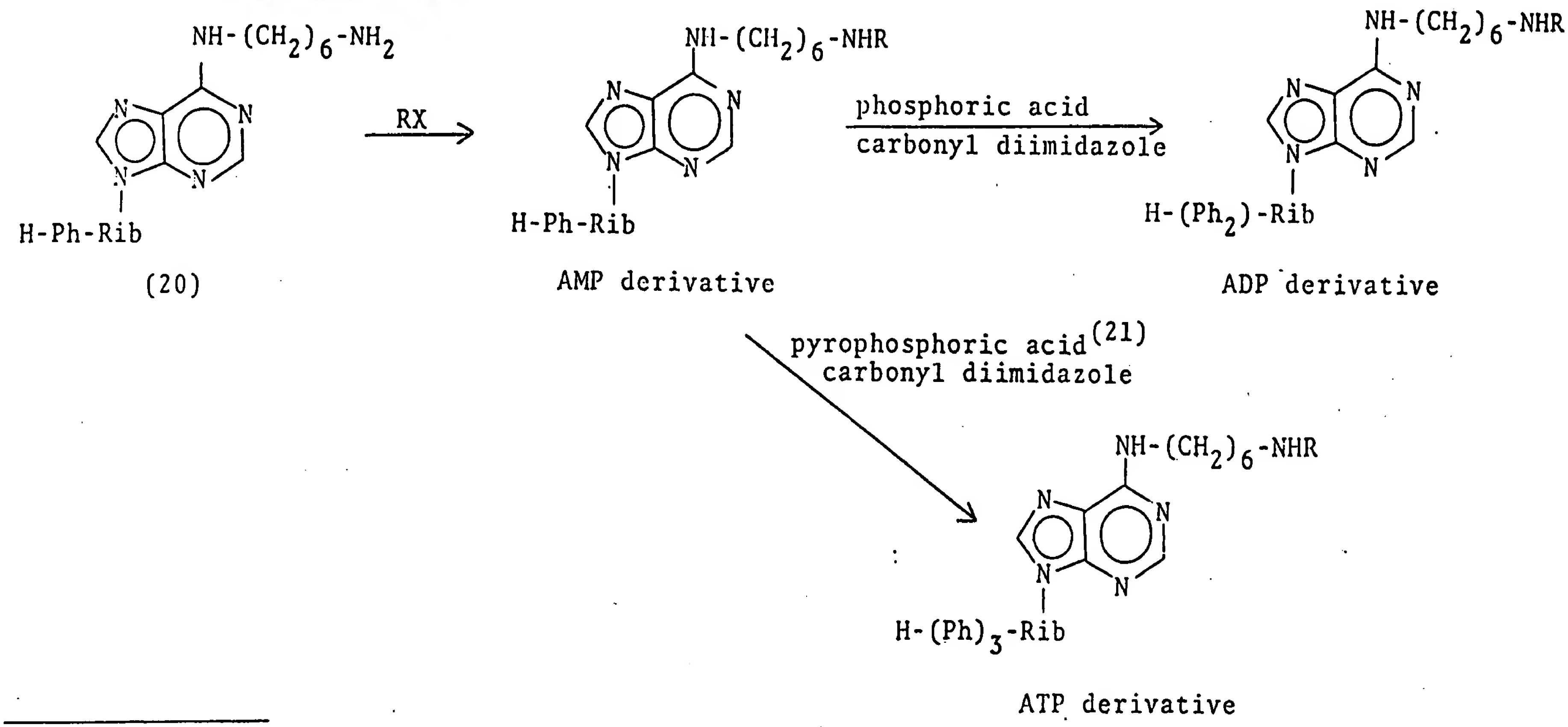
(18) Hughes, N.A., et al., supra.

3-position derivative of NADP



(19) Hughes, N.A., et al., supra.

6-position derivatives of AP



(20) Guilford, H., et al., supra.
(21) Trayer, I.P., et al., supra.

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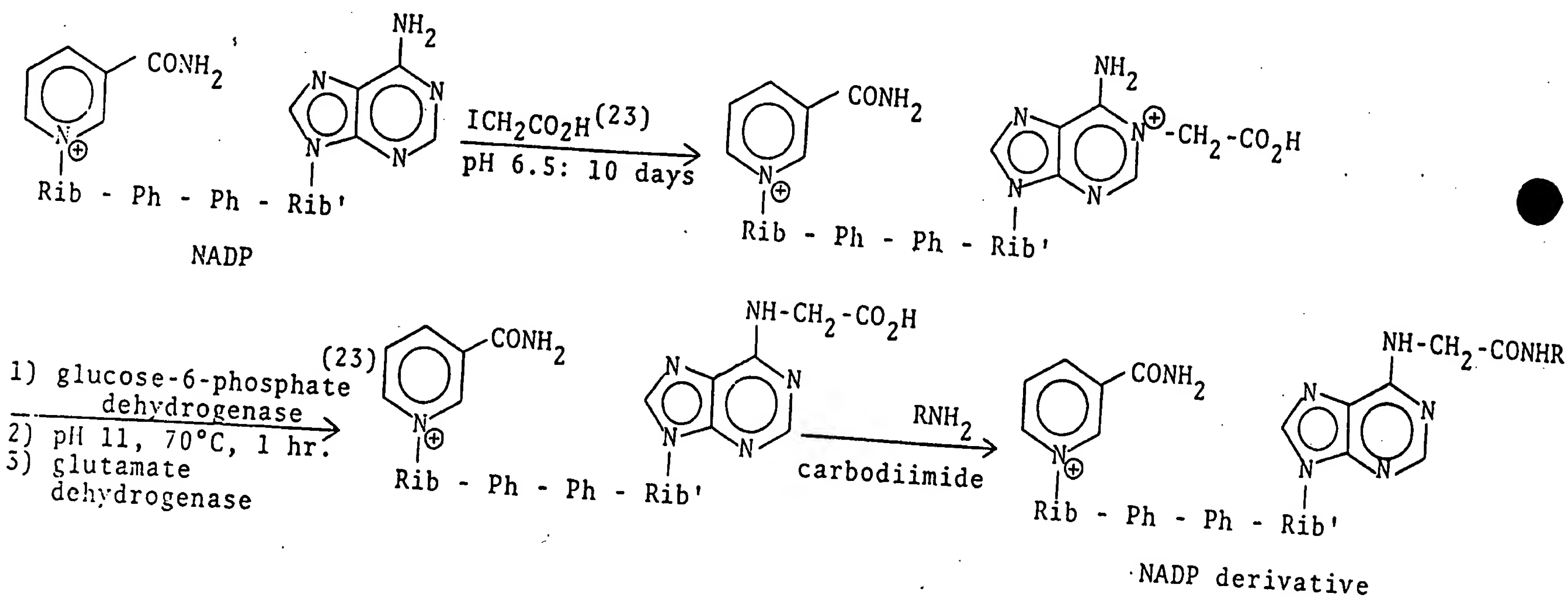
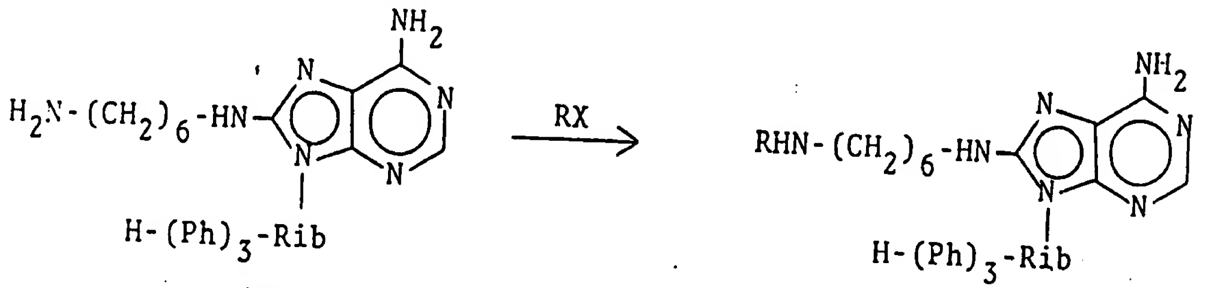
6-position derivative of NADP



(23) Lowe, C.R., and Mosbach, K., supra.

8-position derivatives of AP

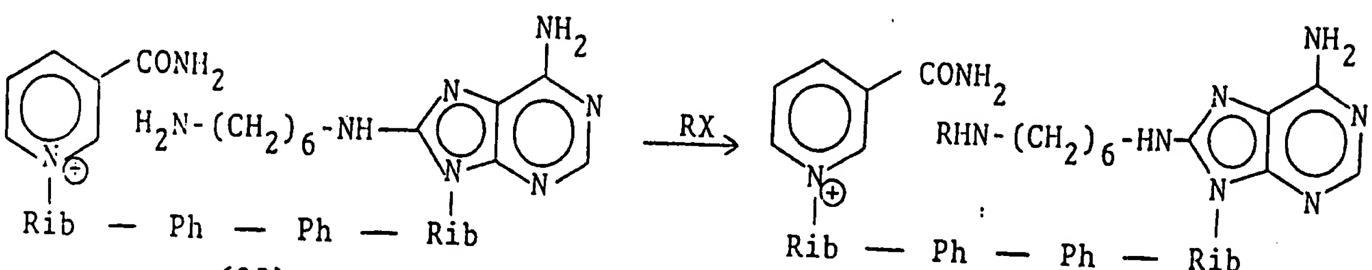


(24)

ATP derivative

(24) Trayer, I.P., et al., supra.

8-position derivative of NAD

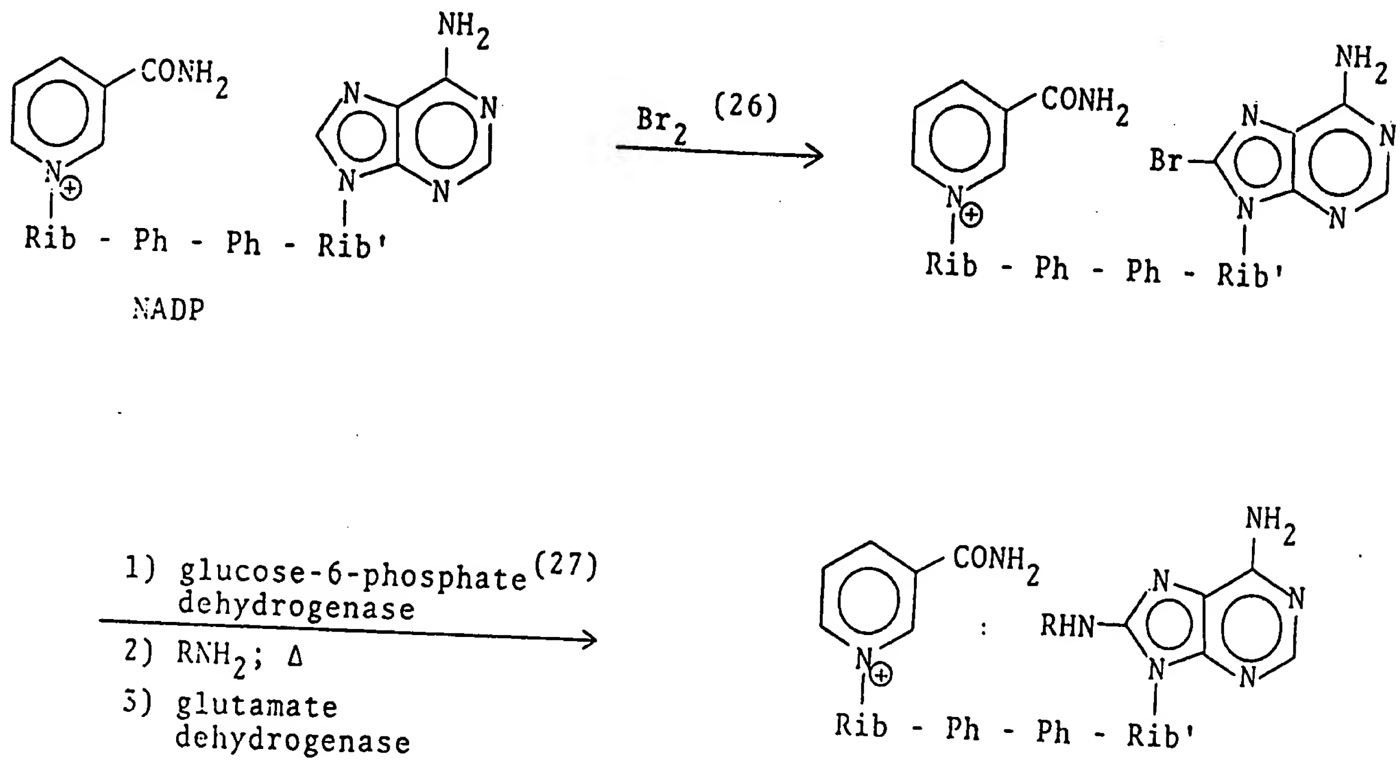


(25)

NAD derivative

(25) Lee, C-Y, et al., *Arch. Biochem. Biophys.* 1C3:561 (1974).

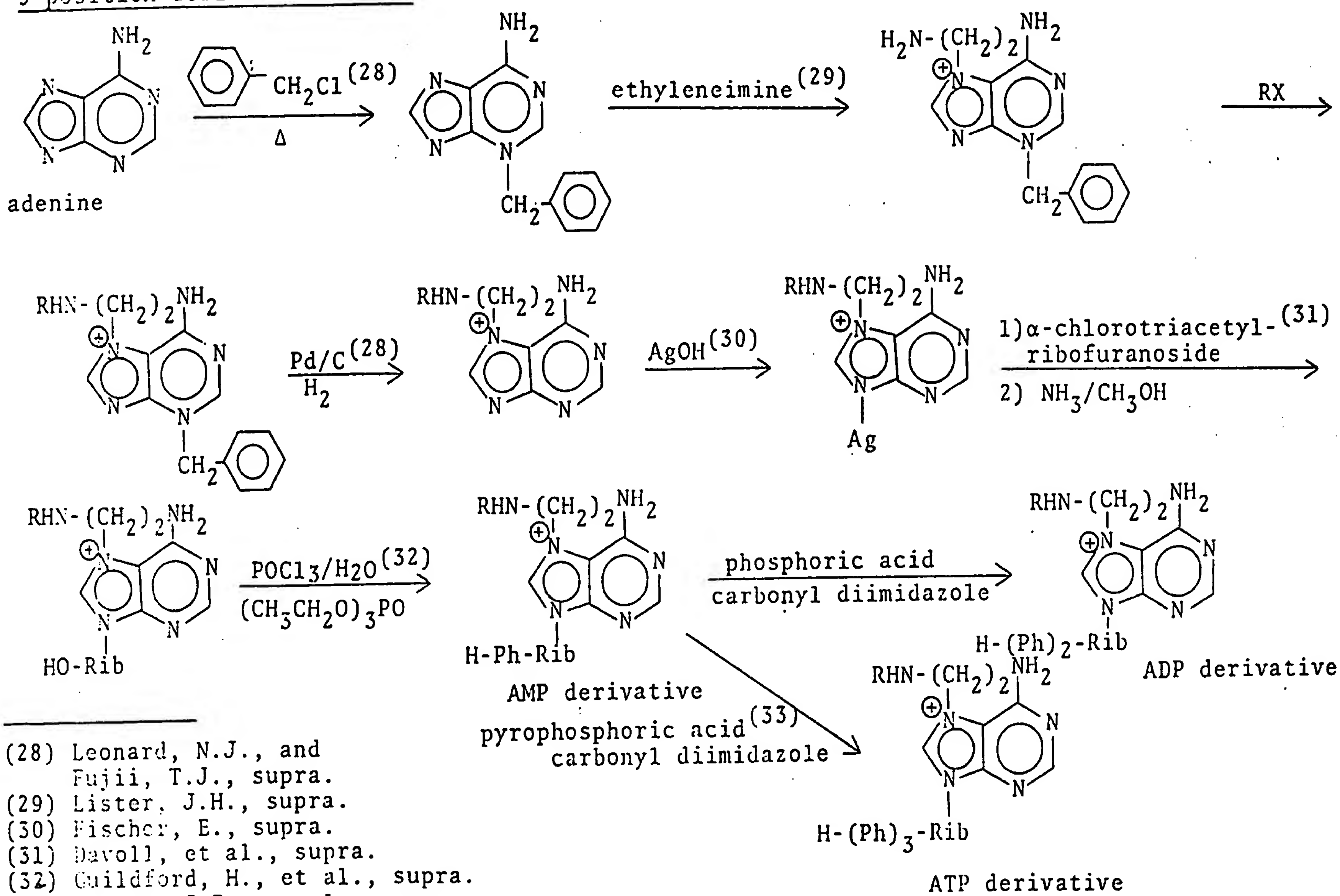
8-position derivative of NADP



NADP derivative

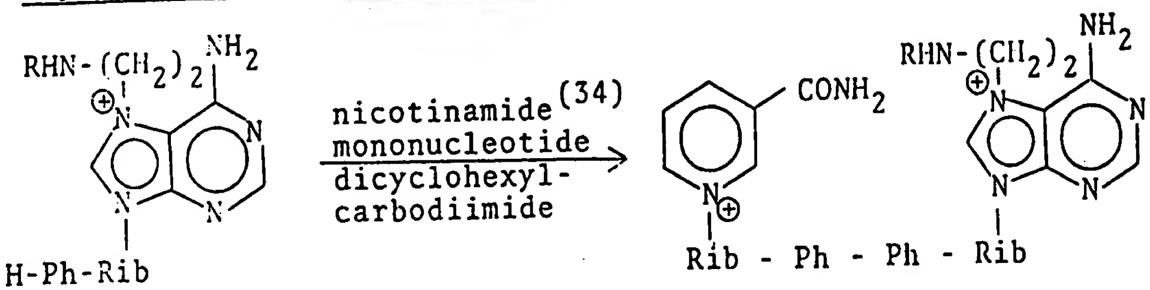
(26) Lee, C-Y, et al., supra.
(27) Lowe, C.R. and Mosbach, K., supra.

9-position derivatives of AP



(28) Leonard, N.J., and Fujii, T.J., supra.
(29) Lister, J.H., supra.
(30) Fischer, E., supra.
(31) Davoll, et al., supra.
(32) Guildford, H., et al., supra.
(33) Trayer, I.P., et al., supra.

9-position derivative of NAD

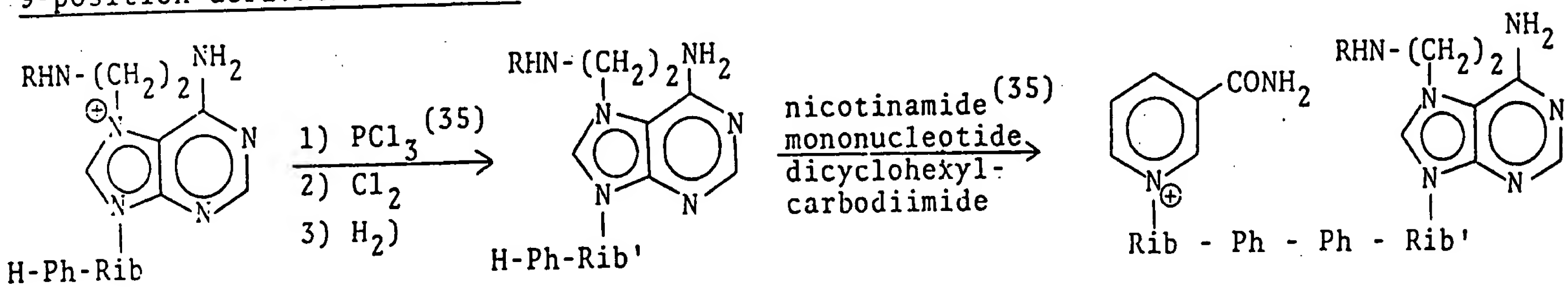


AMP derivative

NAD derivative

(34) Hughes, N.A., et al., supra.

9-position derivative of NADP

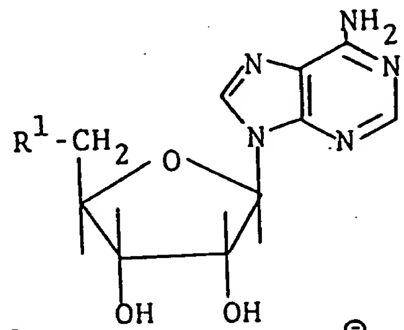


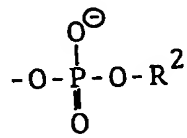
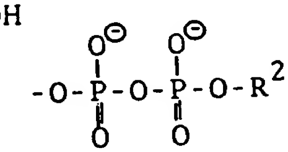
AMP derivative

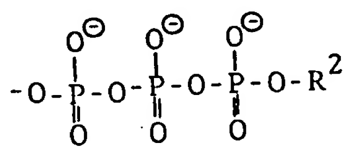
NADP derivative

(35) Hughes, N.A., et al., supra.

In addition to the compounds mentioned above, useful coenzyme-active conjugates include the adenosine phosphates to which are coupled the specific binding substance through the phosphate grouping. Such compounds have the following general formula:



wherein R^1 is  ,  , or



wherein R^2 is -Y-Z; wherein Y is a bond or a bridge group; and wherein Z is a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand. Also, the protonized or acid forms, as well as the salt forms where appropriate, may be used.

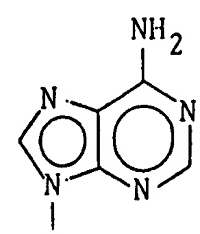
Synthesis of such compounds may be accomplished in a variety of ways. It is contemplated that the synthesis routes which are schematically illustrated below are advantageously followed in the preparation of the useful compounds. The abbreviations used hereinbefore also apply to the illustration to follow.

derivatives of AP

1) $H_2N\text{-}(CH_2)_n\text{-}OH \xrightarrow[\Delta]{\text{phosphoric acid}} H_2N\text{-}(CH_2)_n\text{-}Ph\text{-}H \xrightarrow{RX} RHN\text{-}(CH_2)_n\text{-}Ph\text{-}H$

$n=1\text{-}10$

$\xrightarrow[\text{carbonyl diimidazole}]{\text{adenosine monophosphate [36]}}$



$RHN\text{-}(CH_2)_n\text{-}(Ph)_2\text{-}Rib$

ADP derivative

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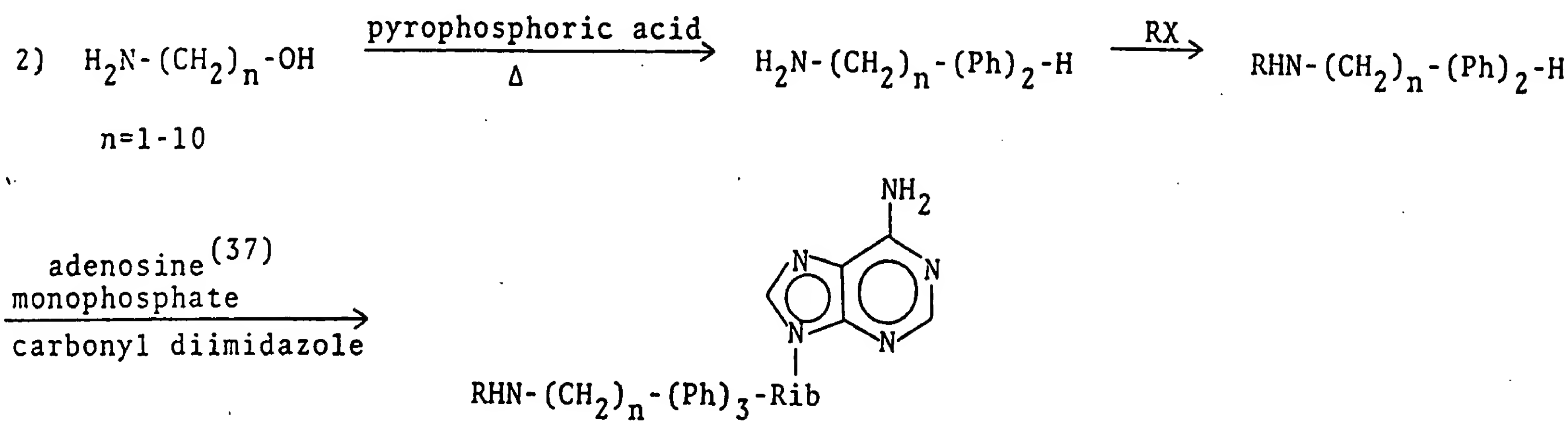
(36) Trayer, I.P., et al., *Biochem. J. 139*:609 (1974).

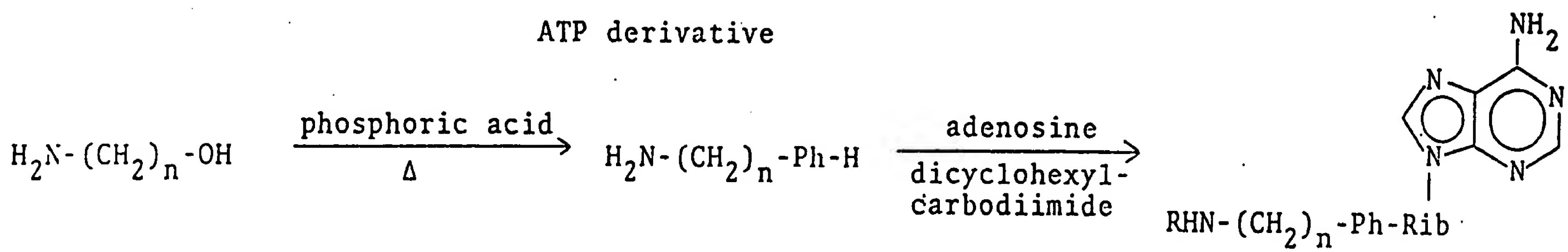
derivatives of AP (Continued)

2)



ATP derivative

3)



AMP derivative

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(37) Trayer, I.P., et al., supra.

In one form of the present invention, the components of the specific binding reaction which are to be combined with the liquid medium suspected of containing the ligand are in a liquid or solid form. In the preferred homogenous assay system, the components are usually in solution or in a solid form capable of ready dissolution in the liquid medium. Since the liquid medium to be tested is normally aqueous in character, the components are generally in a water soluble form, that is, either in aqueous solution or in a water soluble solid form such as a powder or resin. The assay method may be carried out in a standard laboratory vessel such as a test tube with the specific binding reaction components and the components of the reaction system being added thereto in solid or liquid form.

It is also contemplated that one or more of the specific binding reaction components and/or one or more of the components of the predetermined reaction may be incorporated with a carrier. In one aspect, the carrier may be a liquid-holding vessel such as a test tube or capsule containing such component or components in an interior portion thereof, for instance, in the form of a liquid or loose solid or a coating on an interior surface of the vessel. In another aspect, the carrier may be in the form of matrix which is insoluble and porous, and preferably absorbent, relative to the liquid medium to be tested. Such matrix may be in the form of bibulous papers; polymeric films, membranes, fleeces, or blocks; gels; and so forth. In such a form, the device

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inhibitors of such coenzyme-degrading enzymes, for example, chelating agents which operate to deprive the enzymes of essential metal ion activators. As a specific example, NAD-degradable enzymes are found in normal serum and have sufficient enzymatic activity to remove essentially all endogenous NAD activity from isolated serum within a few hours. The degrading activity of such enzymes may be effectively inhibited by addition of a chelating agent such as ethylenediamine tetraacetic acid. Elimination of the degrading activity may also be accomplished by adding a specific enzyme inhibitor. For example, ATP-degrading enzymes may be inhibited by addition of $\beta\gamma$ methylene ATP or $\alpha\beta$ methylene ATP.

The present invention will now be illustrated, but is not intended to be limited, by the following Examples.

EXAMPLE 1

Preparation of nicotinamide 6-(2-aminoethylamino) purine dinucleotide.

Two (2) grams of nicotinamide adenine dinucleotide (NAD) were dissolved in 10 ml of water and 0.6 ml of ethyleneimine was added dropwise, the pH being maintained below 7 by the addition of 1 M perchloric acid. When addition of ethyleneimine was complete, the pH was adjusted to 4.5 and the reaction was incubated at 20-25°C. At 24 hour intervals 0.6 ml of ethyleneimine was added and the pH readjusted to 4.5. After 96 hours, the solution was poured into 10 volumes of acetone at -10°C. The oil which formed was collected, washed with ether, and dissolved in approximately 50 ml of water in a flask.

The resulting solution was adjusted to pH 7.0-7.5 with 1 N sodium hydroxide, and 1 gram of sodium bicarbonate was added. Nitrogen was bubbled through the solution for from 4 to 5 minutes and 1 gram of sodium hydrosulfite was added. The flask was sealed tightly and allowed to stand at room temperature for 45 minutes. The solution was then oxygenated for 15 minutes and adjusted to pH 11.3 with sodium hydroxide. The solution was heated at 75°C for 1 hour. Then the reaction mixture was cooled to room temperature and 0.6 grams of tris-(hydroxymethyl)-aminomethane was added, followed by 5 N hydrochloric acid to adjust the pH to 7.5. To the resulting solution was added 1000 International units of alcohol dehydrogenase and 1 ml of acetaldehyde. The decreasing optical density of the reaction mixture was monitored at 340 nm and when no further decrease was observed, the pH was

adjusted to 3.5. The solution was poured into 10 volumes of acetone at -10°C. The oil which formed was separated and washed with ether, after which it was dissolved in 10 to 15 ml of water.

The resulting solution was introduced into a 2.5x90 cm column of Sephadex G-10, available from Pharmacia AB, Uppsala, Sweden, equilabrated with water. Fractions of 12 ml volume were collected. The wavelength of maximum optical absorption in the ultraviolet region and the optical density at such wavelength were determined for each fraction. Also, the optical density at 340 nm of each fraction after reduction with alcohol dehydrogenase was determined. The fractions which had an optical absorption maximum at 264 nm and had a ratio of optical density at 340 nm to that at 264 nm greater than 0.05 were pooled. The pooled material was concentrated to from 15 to 20 ml on a rotary evaporator and passed through a 2.5x28 cm column of Dowex 1-X8, available from Bio-Rad Laboratories, Richmond, California, equilabrated with water. Additional water was added to wash the pooled material through the column, and 10 ml fractions were collected. The fractions which had an optical absorption maximum at 264 nm and had a ratio of optical density at 340 nm to that at 264 nm greater than 0.1 were pooled.

The pooled material was passed through a 5x45 cm column of Dowex 50-X2, available from Bio-Rad Laboratories, Richmond, California, equilibrated with water. Additional water was added to wash the pooled material through the column and 20 ml fractions were collected. The fractions

which had an optical absorption maximum at 264 nm and had a ratio of optical density at 340 nm to that at 264 nm greater than 0.18 were pooled. The pooled material was concentrated to from 4 to 5 ml and purified by electrophoresis as follows.

The concentrated material was applied to a sheet of Whatman 3MM paper, available from Reeve Angel, Clifton, New Jersey, in a 1 to 2 cm wide strip perpendicular to the direction of current flow. The paper was then wetted with 0.02 M sodium phosphate at pH 6.0. Electrophoresis was conducted according to the Durrum hanging paper method, as described in *Science 121*:829(1955), for 4-7 hours with a potential gradient of about 8.5 volts/cm. The location of the desired pyridine nucleotide derivative was determined by fluorescence developed after spraying a test strip of the paper with 0.5 M sodium cyanide according to the procedure described in *J. Biol. Chem. 191*:447(1951). The area containing the desired derivative was cut out of the paper and extracted with three (3) 50 ml volumes of water. The resulting extracts containing nicotinamide 6-(2-aminoethylamino) purine dinucleotide were pooled, concentrated to from 3 to 4 ml, and stored at -20°C.

EXAMPLE 2

Preparation of nicotinamide adenine dinucleotide -biotin conjugate.

A 16 mg quantity of biotin was suspended in 1 ml of water containing 22 mg of nicotinamide 6-(2-aminoethylamino) purine dinucleotide prepared as in Example 1. A few drops

of 0.1 N sodium hydroxide was added to aid dissolution of the biotin. A 240 mg quantity of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-tolulene sulfonate was added to the resulting solution and brought into solution by dropwise addition of 0.1 N hydrochloric acid. The reaction mixture was allowed to incubate at room temperature for 5 hours and was then poured into 10 ml of acetone at -10°C. The oil which formed was separated, washed twice with from 5 to 10 ml of ether and dissolved in from 1 to 2 ml of water. The resulting material was purified by electrophoresis on paper as in Example 1. Two fluorescent bands appeared after spraying with sodium cyanide, one having migrated toward the cathode and the other toward the anode. The latter band, which contained the NAD-biotin conjugate, was eluted with water and stored at -20°C.

EXAMPLE 3

Preparation of nicotinamide adenine dinucleotide -2,4 dinitrophenyl conjugate.

A 26 mg quantity of sodium bicarbonate was dissolved in 1.5 ml of water containing 23 mg of nicotinamide 6-(2 -aminoethylamino) purine dinucleotide prepared as in Example 1. To the resulting solution was added 3 ml of ethanol containing 17 μl of 2,4 dinitrofluorobenzene. The reaction mixture was stirred at room temperature in the dark for 5 hours after which 45 ml of acetone at -10°C was added thereto. The precipitate which formed was separated, washed twice with 10 ml of acetone, and stirred with 5 ml of water. The yellow soluble material which separated was

purified by electrophoresis on paper as in Example 1 for 5 hours. The band which migrated toward the anode, and which contained the NAD-2,4 dinitrophenyl conjugate, was eluted with water, concentrated to from 3 to 5 ml, and stored at -20°C.

EXAMPLE 4

Preparation of biotin-umbelliferone conjugate.

A reaction mixture was formed by dissolving in 10 ml of dimethylformamide 100 mg of umbelliferone, 167 mg of biotin, and 141 mg of dicyclohexyl carbodiimide. The reaction mixture was incubated at -18°C for about 4 hours, then overnight at 7°C and allowed to stand at room temperature for from 3 to 4 hours. An additional 141 mg of dicyclohexylcarbodiimide was added and the reaction mixture was stirred at 7°C for from 3 to 4 hours and allowed to stand at room temperature overnight. The resulting precipitate was filtered off and discarded. To the filtrate was added 75 ml of ice water, and the resulting mixture was incubated at 0°C for 1 hour. The precipitate which resulted was filtered off and discarded. The filtrate was evaporated to dryness and the residue dissolved in from 3 to 4 ml of methylene chloride. To the resulting solution was added 5 ml of diethylether. The resulting precipitate which comprised the biotin-umbelliferone conjugate was filtered off, dried, and stored at room temperature.

EXAMPLE 5

Effect of avidin and biotin on the enzymatic cycling rate of NAD and NAD-biotin conjugates.

The cycling reaction system used in this Example was based on the following reactions:

(a) NAD-ligand + lactate $\xrightarrow[\text{dehydrogenase}]{\text{lactic}}$

NADH-ligand + pyruvate

(b) NADH-ligand + thiazolyl blue (oxidized) $\xrightarrow{\text{diaphorase}}$

NAD-ligand + thiazolyl blue (reduced)

Eight specific binding reaction mixtures were prepared, each having a total volume of 0.5 ml and containing 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride buffer at pH 7.8 and respectively containing the concentrations and activities indicated in Table 1 of NAD, NAD-biotin conjugate prepared as in Example 2, biotin, and avidin, which latter has an affinity for binding with biotin. One (1) unit of avidin activity is that quantity of avidin capable of binding 1 μg of biotin. The reaction mixtures were incubated at room temperature for from 2 to 3 hours. Each reaction mixture was contacted with an aqueous enzyme/substrate mixture by the addition of 0.1 ml of 1 M lithium lactate, 0.05 ml of 10 mM thiazolyl blue in its oxidized form, and a sufficient quantity of 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride buffer at pH 7.8 containing 0.38 International units of bovine heart lactic dehydrogenase, and 1.5 International units of porcine heart diaphorase to give a total reaction

volume of 1 ml. The relative rate of production of the reduced form of thiazolyl blue was then determined in each of the reaction mixtures by measuring the total change in the optical density in each thereof at 570 nm during a 24 minute period within the first hour after the addition of the enzyme/substrate mixture. The entire procedure was performed in duplicate and the averaged results appear in Table 1.

TABLE 1

reaction	concentration of NAD (nM)	concentration of NAD-biotin conjugate (nM)	concentration of biotin (nM)	avidin activity (units)	average increase in optical density (570 nm)
1	-	-	-	-	0.002
2	220	-	-	-	0.103
3	-	360	-	-	0.079
4	-	-	360	-	0.003
5	220	-	-	0.11	0.103
6	-	360	-	0.11	0.025
7	-	360	360	0.11	0.069
8	-	-	-	0.11	0.001

Reactions 1, 4, and 8 were controls and show that in the absence of NAD and the NAD-biotin conjugate essentially no cycling occurred. The results of reactions 2 and 3 demonstrate that the NAD-biotin conjugate has a significant amount of coenzyme activity relative to native NAD. It can be seen from the results of reactions 3 and 6 that the presence of avidin in the reaction mixture inhibits the formation of thiazolyl blue (reduced form) where the NAD present is conjugated with biotin. By comparing the results of reactions 6 and 7 it can be seen that the presence of free biotin reduces the amount of inhibition of thiazolyl blue (reduced form) formation in proportion to the concentration of biotin in the reaction mixture.

It was thus demonstrated in this Example that the activity of the NAD in the NAD-biotin conjugate relative to the cycling reaction system was decreased in the presence of avidin and that the magnitude of such decrease in activity was reduced by the additional presence of biotin.

EXAMPLE 6

Direct binding-cycling assay for avidin; effect of varying levels of avidin on the cycling rate.

The cycling reaction system used in this Example was the same as that diagrammed in Example 5. Seven specific binding reaction mixtures were prepared, each having a total volume of 0.6 ml and each containing 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride buffer at pH 7.8 and 250 nM NAD-biotin conjugate prepared as in Example 2. Six of the reaction mixtures also contained avidin in the amounts indicated in Table 2.

The reaction mixtures were incubated at room temperature for from 2 to 3 hours. Each reaction mixture was contacted with an aqueous enzyme/substrate mixture by the addition of 0.1 ml of 1 M lithium lactate, 0.05 ml of 10 mM thiazolyl blue in its oxidized form, and a sufficient quantity of 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride buffer at pH 7.8 containing 0.38 International units of bovine heart lactic dehydrogenase and 1.5 International units of porcine heart diaphorase to give a total reaction volume of 1 ml. The relative rate of production of the reduced form of thiazolyl blue was then determined in each of the reaction mixtures by measuring the total change in the optical density in each thereof at 570 nm during a 24 minute period within the first hour after the addition of the enzyme/substrate mixture. The ratio, expressed as percent, of the change in optical density in each reaction mixture containing avidin to that in the reaction mixture not containing avidin was calculated and is referred to in Table 2 and Figure 1 as the relative cycling rate. The results appear in Table 2 and in graphical form in Figure 1 of the drawing.

TABLE 2

reaction mixture	amount of avidin added (units)	relative cycling rate (%)
1	0.000	100
2	0.005	96
3	0.010	93
4	0.045	84
5	0.090	68
6	0.120	51
7	0.180	8

It was demonstrated in this Example that the relative cycling rate of the cycling reaction system, and thus the activity of the NAD in the NAD-biotin conjugate, was an inverse function of the amount of avidin present in the specific binding reaction mixture. The present invention therefore provides a test composition and method for quantitatively determining the presence of the ligand avidin in a liquid medium using a direct binding-cycling assay technique.

EXAMPLE 7

Competitive binding-cycling assay for biotin; effect of varying levels of biotin on the cycling rate.

The cycling reaction system used in this Example was the same as that diagrammed in Example 5. Seven specific binding reaction mixtures were prepared, each having a total volume of 0.45 ml and each containing 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride buffer at pH 7.8 and 180 nM NAD-biotin conjugate prepared as in Example 2. Six of the reaction mixtures, i.e. nos. 1 through 6 in Table 3, additionally contained 0.11 units of avidin. Also, biotin, at the concentrations indicated in Table 3, was included in five of the six reaction mixtures containing avidin, i.e. mixtures 2 through 6 in Table 3.

The reaction mixtures were incubated at room temperature for from 2 to 3 hours. Each reaction mixture was contacted with an aqueous enzyme/substrate mixture by the addition of 0.1 ml of 1 M lithium lactate, 0.05 ml of 10 mM thiazolyl blue in its oxidized form and a sufficient quantity of 0.12 M N,N bis-2-hydroxyethylglycine hydrochloride

buffer at pH 7.8 containing 0.38 International units of bovine heart lactic dehydrogenase and 1.5 International units of porcine heart diaphorase to give a total reaction volume of 1 ml. The relative rate of production of the reduced form of thiazolyl blue was then determined in each of the reaction mixtures by measuring the total change in the optical density in each thereof at 570 nm during a 24 minute period within the first hour after the addition of the enzyme/substrate mixture. The ratio, expressed as percent, of such change in optical density in each reaction mixture containing biotin to that in the reaction mixture not containing either biotin or avidin was calculated and is referred to in Table 3 and Figure 2 as the relative cycling rate. The results appear in Table 3 and in graphical form in Figure 2 of the drawing.

TABLE 3

reaction mixture	concentration of biotin (nM)	relative cycling rate (%)
1	0	8
2	80	22
3	160	35
4	320	63
5	400	80
6	800	92

It was demonstrated in this Example that the relative cycling rate of the cycling reaction system, and thus the activity of the NAD in the NAD-biotin conjugate, was a

direct function of the amount of biotin present in the specific binding reaction mixture. The present invention therefore provides a test composition and method for quantitatively determining the presence of the ligand biotin in a liquid medium using a competitive binding-cycling assay technique.

EXAMPLE 8

Direct binding-cycling assay for antibody to 2,4 dinitrophenyl and derivatives thereof.

The cycling reaction system used in this Example was the same as that described in Example 5. Eight 0.6 ml specific binding reaction mixtures were prepared, each containing 0.12 M N,N bis-hydroxyethylglycine hydrochloride buffer at pH 7.8 and respectively containing the amounts and concentrations indicated in Table 4 of NAD, NAD-2,4 dinitrophenyl conjugate prepared as in Example 3, antiserum to 2,4 dinitrophenyl, and nicotinamide mononucleotide (NMN). The reaction mixtures were incubated at room temperature for from 3 to 4 hours. Each reaction mixture was contacted with an aqueous enzyme/substrate mixture by the addition of 0.1 ml of 1M lithium lactate, 0.05 ml of 10 mM thiazolyl blue in its oxidized form, and a sufficient quantity of 0.12 M N,N bis-hydroxyethylglycine hydrochloride buffer at pH 7.8 containing 0.38 International units of bovine heart lactic dehydrogenase and 1.5 International units of porcine heart diaphorase to give a total reaction volume of 1 ml. The relative rate of production of the reduced form of thiazolyl

blue was determined in each of the reaction mixtures by measuring the total change in the optical density in each thereof at 570 nm during a 24 minute period within the first hour after the addition of the enzyme/substrate mixture. The entire procedure was performed in duplicate and the averaged results appear in Table 4.

TABLE 4

reaction	concentration of NAD (μM)	concentration of NAD-2,4 dinitrophenyl conjugate (nM)	concentration of NMN (μM)	amount of antiserum (μl)	average increas optical dens (570 nm)
1	-	-	-	-	0.005
2	-	290	-	-	0.164
3	1.75	-	-	-	0.608
4	1.75	-	50	-	0.699
5	1.75	-	-	100	0.275
6	1.75	-	50	100	0.648
7	-	290	-	100	0.021
8	-	290	50	100	0.037

Reaction 1 was a control and shows that in the absence of NAD and the NAD-2,4 dinitrophenyl conjugate essentially no cycling occurred. From the results of reaction 2 it is demonstrated that the NAD-2,4 dinitrophenyl conjugate is active in the enzymatic cycling system. The results of reactions 3 and 5 indicate that the presence of antibody to 2,4 dinitrophenyl inhibits the cycling of NAD. As shown by the results of reaction 6, such inhibition is reversed by addition of NMN. From the results of reactions 3 and 4 it is seen that the cycling rate in the presence of NMN is about 15% greater than in its absence. This result is probably due to contamination by extraneous NAD because other measurements have shown that NMN does not influence the cycling rate in the absence of antibody. Nevertheless, the antiserum contains some activity with respect to NAD itself which activity is inhibited by the presence of NMN.

It was thus demonstrated in this Example that the activity of the NAD in the NAD-2,4 dinitrophenyl conjugate relative to the cycling reaction system was decreased in the presence of antibody to 2,4 dinitrophenyl. The present invention therefore provides a test composition and method for determining the presence of the ligand antibody to 2,4 dinitrophenyl in a liquid medium using a direct binding cycling assay technique.

EXAMPLE 9

Competitive binding-cycling assay for 2,4-dinitrobenzene and derivatives thereof; effect of various levels of N (2,4 dinitrophenyl)-6-aminocaproate on the cycling rate.

The cycling reaction system used in this Example was the same as that diagrammed in Example 5. Seven specific binding reaction mixtures were prepared, each having a total volume of 0.6 ml and each containing 0.12 M N,N bis-hydroxyethylglycine hydrochloride buffer at pH 7.8, 300 nM NAD-dinitrophenyl conjugate prepared as in Example 3, and 50 μM nicotinamide mononucleotide. Six of the seven reaction mixtures, i.e. nos. 1 through 6 in Table 5, also contained an amount of antibody to 2,4 dinitrophenyl sufficient to inhibit the cycling rate of the other reaction mixture by 85 percent. N (2,4 dinitrophenyl)-6-aminocaproate, a derivative of 2,4 dinitrobenzene prepared by the method described in *Biochem. J. 42*:287(1948), was also included in five of the six antibody-containing reaction mixtures, i.e. nos. 2 through 6 in Table 5, at the concentrations indicated in said Table.

The reaction mixtures were incubated at room temperature for about 4 hours. Each reaction mixture was contacted with an aqueous enzyme/substrate mixture by the addition of 0.1 ml of 1 M lithium lactate, 0.05 ml of 10 mM thiazolyl blue in its oxidized form, and a sufficient quantity of 0.12 M N,N bis-hydroxyethylglycine hydrochloride buffer at pH 7.8 containing 0.38 International units of bovine heart lactic dehydrogenase and 1.5 International units of porcine heart diaphorase to give a total reaction volume of 1 ml. The relative rate of production of the reduced form of thiazolyl blue was determined in each of the reaction mixtures by measuring the total change in the optical density in each thereof at 570 nm during a 24 minute period within the first hour after the addition of the enzyme/substrate mixture.

The ratio, expressed as percent, of such change in optical density in each reaction mixture containing N (2,4 dinitrophenyl)-6-aminocaproate to that in the reaction mixture containing neither N (2,4 dinitrophenyl)-6-aminocaproate nor antibody to 2,4 dinitrophenyl was calculated and is referred to in Table 5 and Figure 3 as the relative cycling rate. The results appear in Table 5 and in graphical form in Figure 3 of the drawing.

TABLE 5

reaction mixture	concentration of N (2,4 dinitrophenyl) -6-aminocaproate (nM)	relative cycling rate (%)
1	0	16
2	17	19
3	42	30
4	83	35
5	166	41
6	415	76

It was thus demonstrated in this Example that the relative cycling rate of the cycling reaction system, and thus the activity of the NAD in the NAD-dinitrophenyl conjugate, was a direct function of the amount of N (2,4 dinitrophenyl)-6-aminocaproate present in the specific binding reaction mixture. The present invention therefore provides a test composition and method for quantitatively determining the presence of the ligand N (2,4 dinitrophenyl) -6-aminocaproate in a liquid medium using a competitive binding-cycling assay technique.

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was centrifuged at 1500xg for 10 minutes and the pellet was discarded. The light-generating solution was then prepared within 5 minutes of use by combining 75 µl of the reagent mixture, 5 µl of the dodecanal emulsion, and 20 µl of the luciferase solution.

To detect the light produced by reaction (e) a photometer was constructed consisting of a photodetector and a 6 x 50 mm cuvette mounted within a light integrating sphere such that light generated in the cuvette was reflected onto the photodetector. The electronic signal produced by the photodetector was passed to a strip chart recorder. The peak light intensity, as the term is used herein, was measured from the recorder trace and assigned arbitrary units based on the chart paper divisions.

Nine specific binding reaction mixtures were prepared, each having a total volume of 0.2 ml and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0, 0.01 M semicarbazide hydrochloride, and respectively the amounts or concentrations indicated in Table 6 of ethanol, NAD, NAD-biotin conjugate prepared as in Example 2, biotin, and avidin. The reaction mixtures were incubated at room temperature for 10 minutes. Then, 0.025 International units of alcohol dehydrogenase was added to each reaction mixture to initiate a reduction reaction. Semicarbazide combines with the acetaldehyde produced in reaction (c) to form a semicarbazone and thus to drive reaction (c) in the desired direction.

The reaction mixtures were incubated at room temperature for about 30 minutes. A 10 μl volume of each reaction mixture was then injected into a separate cuvette mounted in the photometer previously described and containing 100 μl of the previously prepared light-generating solution which had been pre-incubated at 28°C for from 2 to 3 minutes. The results appear in Table 6.

TABLE 6

reaction	concentration of ethanol (M)	concentration of NAD (nM)	concentration of NAD-biotin conjugate (nM)	concentration of biotin (nM)	avidin activity (units)	peak light intensity
1	-	375	-	-	-	2
2	0.6	375	-	-	-	136
3	0.6	375	-	-	0.054	140
4	-	-	343	-	-	2
5	0.6	-	343	-	-	56
6	0.6	-	343	-	0.054	15
7	-	-	343	200	0.054	5
8	0.6	-	343	200	0.054	32
9	0.6	-	343	200	-	52

Reactions 1, 4, and 7 were controls and show that in the absence of ethanol essentially no reaction occurred. The result from reaction 5 demonstrates that the NAD-biotin conjugate is active in the bioluminescence reaction system. It can be seen from the results of reactions 5 and 6 that the presence of avidin in the reaction mixture inhibits the amount of light produced. From a comparison of the results of reactions 6 and 8 it is seen that the presence of free biotin reduces the amount of inhibition of light production as the concentration of biotin increases in the reaction mixture. Reactions 2 and 3 demonstrate that avidin does not inhibit the activity of free NAD and reactions 5 and 9 show that the presence of biotin alone does not affec the activity of the NAD-biotin conjugate.

It was thus demonstrated in this Example that the activity of the NAD in the NAD-biotin conjugate relative to the bioluminescense reaction system was decreased in the presence of avidin and that the magnitude of such decrease in activity was reduced by the additional presence of biotin.

EXAMPLE 11

Competitive binding-bioluminescence assay for biotin; effect of varying levels of biotin on the peak light intensity produced.

The bioluminescence reaction system used in this Example was the same as that diagrammed in Example 10. Seven specific binding reaction mixtures were prepared, each having a total volume of 0.2 ml and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0, 0.6 M

ethanol, 0.01 M semicarbazide hydrochloride, 343 nM NAD-biotin conjugate prepared as in Example 2, 0.025 International units of alcohol dehydrogenase, and 0.0[illegible]5 units of avidin. Biotin was added to six of the seven reaction mixtures, i.e. nos. 2 through 7 in Table 7, in the concentrations indicated in said Table. The order and manner of addition was the same as in Example [illegible].

The reaction mixtures were incubated at room temperature for about 30 minutes. A 10 µl volume of each reaction mixture was injected into a separate cuvette mounted in the photometer described in Example 10 and containing 100 µl of a light-yielding solution prepared in the manner described in Example 10 and pre-incubated at 28°C for from 2 to 3 minutes. The entire procedure was run in duplicate, and the averaged results appear in Table 7 and in graphical form in Figure 4 of the drawing.

TABLE 7

reaction mixture	concentration of biotin (nM)	average peak light intensity
1	0	36
2	[illegible]	44
3	50	57
4	100	79
5	150	90
6	200	97
7	300	104

It was thus demonstrated in this Example that the magnitude of the peak light intensity produced by the bioluminescence reaction system, and thus the activity of the NAD in the NAD-biotin conjugate, was a direct function of the amount of biotin present in the specific binding reaction mixture. The present invention therefore provides a test composition and method for quantitatively determining the presence of the ligand biotin in a liquid medium using a competitive binding-bioluminescence assay technique.

EXAMPLE 12

Competitive binding-bioluminescence assay for 2,4 dinitrobenzene and derivatives thereof; effect of various levels of N (2,4 dinitrophenyl)-6-aminocaproate on the peak light intensity produced.

The bioluminescence reaction system used in this Example was the same as that diagrammed in Example 10. Seven specific binding reaction mixtures were prepared, each having a total volume of 0.1 ml and each containing 0.1 M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0, 0.01 M semicarbazide hydrochloride, 0.6 M ethanol, 35 μM nicotinamide mononucleotide, and 367 nM NAD-dinitrophenyl conjugate prepared as in Example 3. N (2,4 dinitrophenyl)-6-aminocaproate was added to six of the seven reaction mixtures, i.e. nos. 2 through 7 in Table 8, at the concentrations indicated in said Table, and to each of said six reaction mixtures was also added an amount of antibody to 2,4 dinitrophenyl sufficient to reduce the peak light intensity produced to 39% of that produced in the absence of N (2,4 dinitrophenyl)-6-aminocaproate and antibody to 2,4 dinitrophenyl.

The reaction mixtures were incubated at room temperature for 3 hours. Then, 0.025 International units of alcohol dehydrogenase was added to each reaction mixture to initiate a reduction reaction. The reaction mixtures were then incubated at room temperature for about 30 minutes. A 10 µl volume of each reaction mixture was injected into a separate cuvette mounted in the photometer described in Example 10 and containing 100 µl c a light-generating solution prepared in the manner described in Example 10 and pre-incubated at 28°C for from 2 to 3 minutes. The entire procedure was run in duplicate, and the averaged results appear in Table 8 and in graphical form in Figure 5 of the drawing.

TABLE 8

reaction mixture	concentration of N (2,4 dinitrophenyl) -6-aminocaproate µM)	average peak light intensity
1	0.00	14
2	0.125	17
3	0.25	20
4	0.50	22
5	0.75	24
6	1.00	27
7	1.50	28

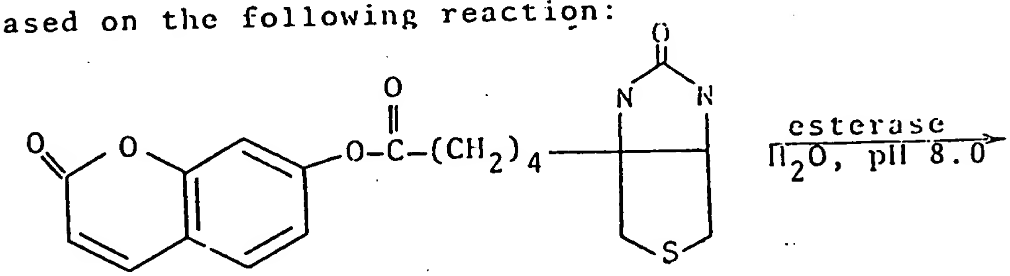
It was thus demonstrated in this Example that the magnitude of the peak li... intensity produced by the bioluminescence reaction system, and thus the activity of the

NAD in the NAD-2,4 dinitrophenyl conjugate, was a direct function of the amount of N (2,4 dinitrophenyl)-6-aminocaproate present in the specific binding reaction. The present invention therefore provides a test composition and method for quantitatively determining the presence of the ligand N (2,4 dinitrophenyl)-6-aminocaproate in a liquid medium using a competitive binding-bioluminescense assay technique.

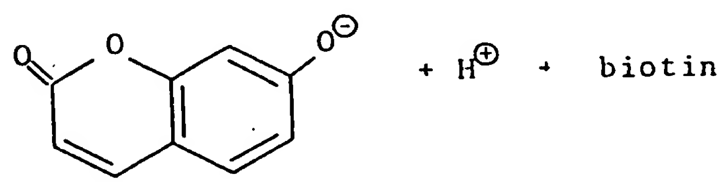
EXAMPLE 13

Specific binding assays for biotin and avidin employing an enzyme substrate as labeling substance.

The specific binding assay system used in this Example was based on the following reaction:



umbelliferone-biotin conjugate
(maximum fluorescence at 378 nm)



(maximum fluorescence at 448 nm)

Ten specific binding reaction mixtures were prepared, each having a total volume of 0.3 ml and each containing 0. M tris-(hydroxymethyl)-aminomethane hydrochloride buffer at pH 8.0 and the respective amounts or concentrations of

umbelliferone-biotin conjugate prepared as in Example 4, biotin, and avidin indicated in Table 9. The reaction mixtures were incubated at room temperature for from 1 to 3 minutes. Reaction mixtures nos. 2 through 10 in Table 9 each also contained 0.26 International units of bovine liver carboxylate hydrolase (esterase). The relative reaction rate in each of the reaction mixtures was then determined by monitoring the fluorescence produced by each thereof at 448 nm with a Model 111 Turner flu .ometer (available from G.K. Turner Assoc., 2524 Pulgas Street, Palo Alto, California) set for excitation at 364 nm. The electronic signal produced by the fluorometer was passed to a strip chart recorder, and the amount of fluorescence produced per minute was measured from the recorder trace and assigned arbitrary units based on the chart paper divisions. The results appear in Table 9.

TABLE 9

reaction	esterasc (I.U.)	concentration of umbelliferone-biotin conjugate (nM)	concentration of biotin (nM)	avidin activity (units)	change in fluorescence/min.
1	-	730	-	-	0.000
2	0.26	365	-	-	0.077
3	0.26	730	-	-	0.145
4	0.26	730	-	0.0022	0.103
5	0.26	730	-	0.022	0.058
6	0.26	730	-	0.055	0.000
7	0.26	730	-	0.033	0.027
8	0.26	730	-	0.033	0.026
9	0.26	730	670	0.033	0.057
10	0.26	730	1340	0.033	0.115

Reaction 1 was a control and shows that in the absence of esterase no reaction occurs. The results of reactions 2 and 3 demonstrate that the umbelliferone-biotin conjugate was active in the enzymatic reaction, and comparing such results to those of reactions 4 through 8 demonstrates that the presence of avidin inhibits the reaction rate in proportion to the amount of avidin in the reaction mixture. Comparing the results of reactions 8, 9, and 10 shows that the amount of inhibition of the reaction rate by avidin is a direct function of the amount of biotin present in the reaction mixture.

It was thus demonstrated in this Example that the rate of fluorescence produced by the esterase reaction, and thus the substrate activity of the umbelliferone-biotin conjugate, was decreased by the presence of avidin and that the magnitude of such decrease in activity was reduced by the presence of biotin. The present invention therefore provides a test composition and method for determining the presence of the ligands biotin and avidin in a liquid medium using a specific binding assay technique employing an enzyme substrate as the labeling substance.

WHAT IS CLAIMED IS:

1. A method of assaying a liquid medium for a ligand which method comprises the steps of:

(1) contacting said medium

(a) with a conjugate comprising a substance which exhibits given reactant activity as a constituent of a predetermined reaction, said substance being coupled to a specific binding substance,

which latter is

(i) said ligand,

(ii) a specific binding analog of said ligand, or

(iii) a specific binding partner of said ligand,

(b) and, if said specific binding substance is said ligand or said specific binding analog thereof, with a specific binding partner of said ligand; and

(2) thereafter assessing any resulting change in said reactant activity as an indication of the presence of said ligand in said liquid medium.

2. A method as in Claim 1 wherein the assessment of change in said reactant activity comprises the steps of:

contacting said medium with at least one second substance which forms, with said first-mentioned substance, said predetermined reaction, and

comparing a characteristic of the resulting reaction to that of said predetermined reaction in a liquid medium devoid of said ligand, any difference therebetween being an indication of a change in said reactant activity.

3. A method as in Claim 1 wherein assessment of change in said reactant activity comprises the steps of:

contacting said medium with at least one second substance which forms, with said first-mentioned substance, said predetermined reaction, and

comparing a characteristic of the resulting reaction to that of said predetermined reaction in liquid media containing known amounts of said ligand.

4. A method as in Claim 3 wherein said characteristic is reaction rate.

5. A method as in Claim 3 wherein said predetermined reaction includes a cyclic reaction.

6. A method as in Claim 5 wherein a cycled material in said cyclic reaction is said first-mentioned substance.

7. A method as in Claim 5 wherein said cyclic reaction is an exponential cyclic reaction.

8. A method as in Claim 7 wherein a cycled material in said cyclic reaction is said first-mentioned substance.

9. A method as in Claim 3 wherein said predetermined reaction includes a luminescent reaction.

10. A method as in Claim 1 wherein said specific binding substance in said conjugate is said ligand or a specific binding analog of said ligand and wherein said specific binding partner is contacted substantially simultaneously with said conjugate and said medium.

11. A method as in Claim 10 wherein said specific binding partner is contacted with said medium and said conjugate in an amount less than that capable of binding with all of said ligand in said medium and all of said ligand or analog thereof in conjugated form during the time that said specific binding partner, said conjugate, and said medium are in contact prior to step (2).

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16. A method as in Claim 15 wherein the amount of said ligand or analog thereof in conjugated form is in excess of that capable of binding with the amount of said specific binding partner present during the time that said conjugate and said specific binding partner are in contact prior to contact with said medium, and wherein the amount of said ligand or analog thereof in conjugated form which becomes bound to said specific binding partner is in excess of that capable of being displaced by all of said ligand in said liquid medium during the time that said specific binding partner and said medium are in contact prior to step (2).

17. A method as in Claim 1 wherein said specific binding substance in said conjugate is said ligand or a specific binding analog of said ligand and wherein said conjugate and said specific binding partner are in the form of a complex, said specific binding substance in said conjugate and said specific binding partner being reversibly bound to each other.

18. A method as in Claim 17 wherein the amount of said ligand or analog thereof in conjugated form is in excess of that capable of being displaced by all of said ligand in said liquid medium during the time that said complex and said medium are in contact prior to step (2).

19. A method as in Claim 1 wherein said ligand is selected from the group consisting of antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites, and pharmacological agents, and their receptors and binding substances.

20. A method as in Claim 1 wherein said liquid medium is a biological fluid.

21. A method as in Claim 1 wherein said predetermined reaction is enzyme-catalyzed.

22. A method as in Claim 21 wherein said first-mentioned substance is an enzyme substrate.

23. A method as in Claim 21 wherein said first-mentioned substance is a coenzyme.

24. A method as in Claim 21 wherein said first-mentioned substance is a nucleotide coenzyme.

25. A method as in Claim 21 wherein said first-mentioned substance is selected from the group consisting of the adenosine phosphates, nicotinamide adenine dinucleotide and reduced forms thereof, and nicotinamide adenine dinucleotide phosphate and reduced forms thereof.

26. A method as in Claim 21 wherein said first-mentioned substance is nicotinamide adenine dinucleotide or a reduced form thereof.

27. A method as in Claim 21 wherein said first-mentioned substance has a molecular weight of less than 9000.

28. A method as in Claim 21 wherein said first-mentioned substance has a molecular weight of less than about 5000.

29. A method as in Claim 21 wherein the assessment of change in said reactant activity comprises the steps of:

contacting said medium with at least one second substance which forms, with said first-mentioned substance, said predetermined reaction, and

comparing a characteristic of the resulting reaction to that of said predetermined reaction in liquid media containing known amounts of said ligand.

30. A method as in Claim 29 wherein said characteristic is reaction rate.

31. A method as in Claim 29 wherein said predetermined reaction includes a cyclic reaction.

32. A method as in Claim 31 wherein a cycled material in said cyclic reaction is said first-mentioned substance.

33. A method as in Claim 32 wherein said characteristic is the cycling rate of said cyclic reaction.

34. A method as in Claim 31 wherein said cyclic reaction is an exponential cyclic reaction.

35. A method as in Claim 34 wherein a cycled material in said cyclic reaction is said first-mentioned substance.

36. A method as in Claim 29 wherein said predetermined reaction includes a luminescent reaction.

37. A method as in Claim 36 wherein said characteristic is the magnitude of the peak light intensity produced.

38. A method as in Claim 29 wherein said first-mentioned substance is an enzyme substrate, said conjugate has fluorescent properties, and a product of said predetermined reaction has fluorescent properties which differ from those of said conjugate.

39. A method as in Claim 37 wherein said characteristic is the rate of fluorescence production.

40. A composition for use in a method of assaying a liquid medium for a ligand which composition comprises (a) a conjugate comprising a substance which exhibits given reactant activity as a constituent of a predetermined reaction, said substance being coupled to a specific binding substance, which latter is (i) said ligand, (ii) a specific binding analog of said ligand, or (iii) a specific binding partner of said ligand, and, if said specific binding substance is said ligand or said specific binding analog thereof, (b) a specific binding partner of said ligand, contact of said composition with a liquid medium containing said ligand causing said first-mentioned substance to exhibit reactant activity as a constituent of said predetermined reaction which is different from said given reactant activity.

41. A composition as in Claim 40 wherein said predetermined reaction is enzyme-catalyzed.

42. A composition as in Claim 41 wherein said first -mentioned substance is an enzyme substrate.

43. A composition as in Claim 41 wherein said first -mentioned substance is a coenzyme.

44. A composition as in Claim 41 wherein said first -mentioned substance is a nucleotide coenzyme.

45. A composition as in Claim 41 wherein said first -mentioned substance is selected from the group consisting of the adenosine phosphates, nicotinamide adenine dinucleotide and reduced forms thereof, and nicotinamide adenine dinucleotide phosphate and reduced forms thereof.

46. A composition as in Claim 41 wherein said first -mentioned substance is nicotinamide adenine dinucleotide or a reduced form thereof.

47. A composition as in Claim 41 wherein said first -mentioned substance has a molecular weight of less than 9000.

48. A composition as in Claim 41 wherein said first -mentioned substance has a molecular weight of less than about 5000.

49. A composition as in Claim 41 which additionally comprises at least one second substance which forms, with said first-mentioned substance, said predetermined reaction.

50. A composition as in Claim 49 wherein said pre-determined reaction includes a cyclic reaction.

51. A composition as in Claim 50 wherein a cycled material in said cyclic reaction is said first-mentioned substance.

52. A composition as in Claim 51 wherein said first -mentioned substance is nicotinamide adenine dinucleotide or a reduced form thereof, and, in order to form said cyclic reaction, said at least one second substance comprises:

(1) one of the following groups of substances

(a) lactaldehyde and alcohol dehydrogenase;

(b) α-ketoglutarate, a substance capable of releasing ammonia upon contact with said liquid medium, and glutamic dehydrogenase;

(c) acetaldehyde and alcohol dehydrogenase;

(d) α-ketoglutarate, a substance capable of releasing carbon dioxide upon contact with said liquid medium, and isocitric dehydrogenase;

(e) dehydroxyacetone phosphate and α-glycerol phosphate dehydrogenase;

(f) pyruvate and lactic dehydrogenase;

(g) 1,3-diphosphoglycerate and glyceraldehyde -3-phosphate dehydrogenase; or

(h) oxaloacetate and malic dehydrogenase; and

(2) one of the following groups of substances

(a) ethanol and alcohol dehydrogenase;

(b) isocitrate and isocitric dehydrogenase;

(c) L-α-glycerol phosphate and α-glycerol phosphate dehydrogenase;

(d) lactate and lactic dehydrogenase;

(e) glyceraldehyde-3-phosphate, phosphate, and glyceraldehyde-3-phosphate dehydrogenase;

(f) propanediol and alcohol dehydrogenase;

(g) malate and malic dehydrogenase; or

(h) glutamate and glutamic dehydrogenase.

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54. A composition as in Claim 51 wherein said first-mentioned substance is flavin mononucleotide or a reduced form thereof, and said at least one second substance comprises nicotinamide adenine dinucleotide phosphate, oxidized cytochrome C, and cytochrome C reductase.

55. A composition as in Claim 51 wherein said first-mentioned substance is flavin adenine dinucleotide or a reduced form thereof, and said at least one second substance comprises D-aminoacid and D-aminoacid oxidase.

56. A composition as in Claim 51 wherein said first-mentioned substance is α-aminoacid or α-ketoacid, and said at least one second substance comprises L-aminoacid oxidase, glutamate, and transaminase.

57. A composition as in Claim 51 wherein said first-mentioned substance is adenosine diphosphate or adenosine triphosphate, and said at least one second substance comprises phosphoenol pyruvate, pyruvate kinase, and adenosine triphosphatase.

58. A composition as in Claim 51 wherein said first-mentioned substance is coenzyme A or succinyl-coenzyme A, and said at least one second substance comprises α-ketoglutarate, nicotinamide adenine dinucleotide, α-ketoglutarate dehydrogenase, phosphate, guanosine diphosphate, and succinic thiokinase.

59. A composition as in Claim 51 wherein said first -mentioned substance is oxidized or reduced glutathione, and said at least one second substance comprises nicotinamide adenine dinucleotide phosphate, glutathione reductase, dehydroascorbate, and dehydroascorbate reductase.

60. A composition as in Claim 51 wherein said first -mentioned substance is ascorbate or dehydroascorbate, and said at least one second substance comprises ascorbate oxidase, oxidized glutathione, and dehydroascorbate reductase.

61. A composition as in Claim 51 wherein said first -mentioned substance is guanosine triphosphate or guanosine diphosphate, and said at least one second substance comprises oxaloacetate, phosphoenol pyruvate kinase, adenosine triphosphate, and nucleoside diphosphate kinase.

62. A composition as in Claim 51 wherein said first -mentioned substance is oxidized or reduced cytochrome C, and said at least one second substance comprises nicotinamide adenine dinucleotide phosphate, cytochrome C reductase, hydrogen peroxide, and cytochrome C peroxidase.

63. A composition as in Claim 51 wherein said first -mentioned substance is oxidized or reduced ferridoxin, and said at least one second substance comprises hydrogen, hydrogenase, nicotinamide adenine dinucleotide phosphate, and pyridine nucleotide reductase.

64. A composition as in Claim 51 wherein said cyclic reaction is an exponential cyclic reaction.

65. A composition as in Claim 64 wherein said first -mentioned substance is adenosine triphosphate or adenosine diphosphate, and said at least one second substance comprises adenosine monophosphate, myokinase, phosphoenol pyruvate, and pyruvate kinase.

66. A composition as in Claim 49 wherein said predetermined reaction includes a luminescent reaction.

67. A composition as in Claim 49 wherein said first -mentioned substance is an enzyme substrate, said conjugate has fluroescent properties, and a product of said predetermined reaction has fluorescent properties which differ from those of said conjugate.

68. A composition as in Claim 40 wherein said ligand is selected from the group consisting of antigens and antibodies thereto; haptens and antibodies thereto; and hormones, vitamins, metabolites, and pharmacological agents. and their receptors and binding substances.

69. A composition as in Claim 40 wherein said conjugate and, if present, said specific binding partner are water soluble.

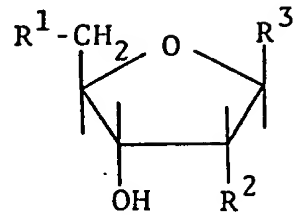
70. A composition as in Claim 40 wherein said conju-
2 gate and, if present, said specific binding partner are in
a dry form.

71. A composition as in Claim 40 wherein said conju-
2 gate is incorporated with a carrier.

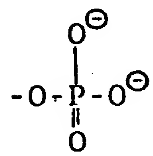
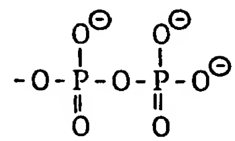
72. A composition as in Claim 71 wherein said carrier
2 is absorbent relative to said liquid medium.

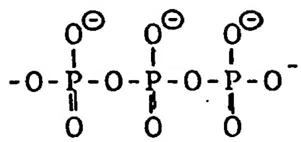
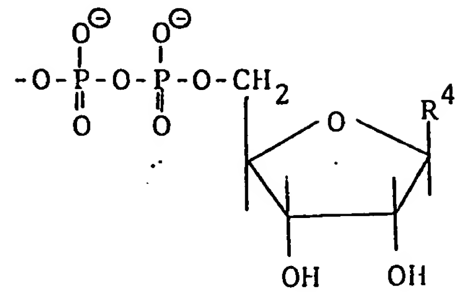
73. A composition as in Claim 71 which includes said
2 specific binding partner, and said binding partner is also
incorporated with said carrier.

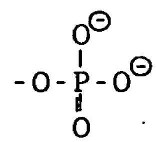
74. A compound having the formula



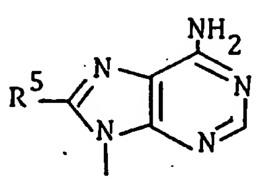
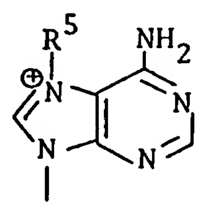
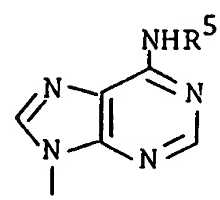
wherein R^1 is

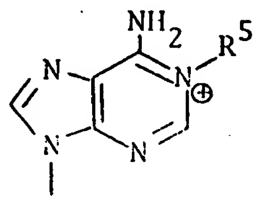
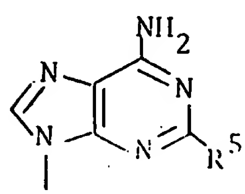
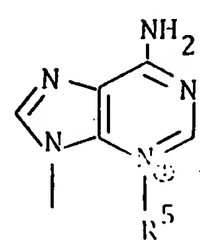
 ,  ,

 , or  ;

wherein R^2 is -OH or  ;

wherein R^3 is

 ,  ,  ,

 ,  or  ;

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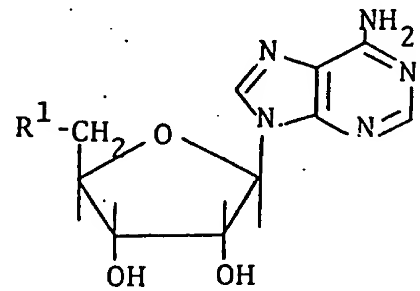
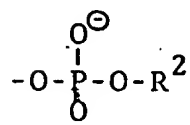
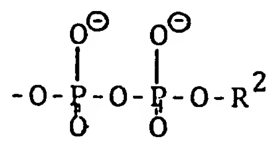
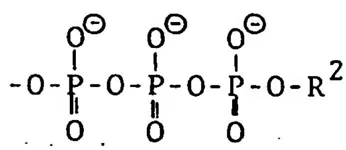
79. A compound as in Claim 78 wherein Y comprises a chain of 1 to 10 carbon atoms and/or heteroatoms.

80. A compound having the formula



wherein R^1 is  ,  , or

 ;

wherein R^2 is -Y-Z; wherein Y is a bond or a bridge group; and wherein Z is a ligand, a specific binding analog of a ligand, or a specific binding partner of a ligand; and the protonized and salt forms of said compound.

81. A compound as in Claim 80 wherein Y is a bridge group having a molecular weight of less than 200.

82. A compound as in Claim 81 wherein Y comprises a chain of 1 to 10 carbon atoms and/or heteroatoms.

ATTORNEY'S DOCKET NUMBER (IF ANY) 11515

DECLARATION AND POWER OF ATTORNEY — for use with sole or joint patent application accompanying or not accompanying the application papers

I/We the undersigned declare that the information (in items 201 to 206 and 301) below is true, that I/we believe that I/we am/are the original, first, and sole/joint inventor(s) of the invention described and claimed in the specification [X] attached [ ] of my/our patent Application Serial Number ______, filed ______, that as to common subject matter of this application and my/our earlier United States application(s), if any, described in item 105 below, I/we do not believe that the same was ever known or used in the United States before my/our invention thereof or patented or described in any printed publication in any country before my/our invention thereof or more than one year prior to such earlier application(s), or in public use or on sale in the United States more than one year prior to such earlier application(s), that the said common subject matter has not been patented(1) before the date of such earlier application(s) in any country foreign to the United States on an application, filed by me/us or my/our legal representatives or assigns more than twelve months prior to such earlier application(s) and that no application for patent(2) on said common subject matter has been filed by me/us or my/our legal representatives or assigns in any country foreign to the United States except those identified in item 600 below, if any; that, as to any subject matter of this application which is not common to any of said earlier application(s) (non-common subject matter), I/we do not know and do not believe that the same was ever known or used in the United States before my/our invention thereof or patented or described in any printed publication in any country before my/our invention thereof or more than one year prior to the date of this application, or in public use or on sale in the United States more than one year prior to the date of this application, and that said non-common subject matter has not been patented(1) in any country foreign to the United States on an application filed by me/us or my/our legal representatives or assigns more than twelve months prior to the date of this application; and that no application for patent(2) on said non-common subject matter has been filed by me/us or my/our legal representatives or assigns in any country foreign to the United States prior to this application except those identified in said item 600.

	FULL NAME OF APPLICANT (INCL. AT LEAST ONE UNABBREVIATED FIRST OR MIDDLE NAME):			
201	LAST: BOGUSLASKI	FIRST: ROBERT	MIDDLE: CHARLES	COUNTRY OF CITIZENSHIP: United States
	RESIDENCE: CITY: Elkhart	STATE (OR FOREIGN COUNTRY): Indiana		
	POST OFFICE ADDRESS: STREET ADDRESS: 52118 (R2) Iris Court	CITY: Elkhart	STATE (OR FOREIGN COUNTRY): Indiana	ZIPCODE: 46514
202	LAST: CARRICO	FIRST: ROBERT	MIDDLE: JOSEPH	COUNTRY OF CITIZENSHIP: United States
	RESIDENCE: CITY: Bremen	STATE (OR FOREIGN COUNTRY): Indiana		
	POST OFFICE ADDRESS: STREET ADDRESS: 65151 Gumwood Road R2	CITY: Bremen	STATE (OR FOREIGN COUNTRY): Indiana	ZIPCODE: 46506
203	LAST: CHRISTNER	FIRST: JAMES	MIDDLE: EDWARD	COUNTRY OF CITIZENSHIP: United States
	RESIDENCE: CITY: Ann Arbor	STATE (OR FOREIGN COUNTRY): Michigan		
	POST OFFICE ADDRESS: STREET ADDRESS: 1563 McIntyre	CITY: Ann Arbor	STATE (OR FOREIGN COUNTRY): Michigan	ZIPCODE: 48105

LISTING OF APPLICANTS CONTINUED ON PAGE 2 HEREOF. [ ] YES [X] NO

301 TITLE OF INVENTION: SPECIFIC BINDING ASSAY METHOD AND COMPOSITION, COMPOUND, AND DEVICE FOR USE THEREIN

CONTINUED ON PAGE 2 HEREOF [ ] YES [ ] NO

600 Earliest foreign application, if any, filed within 12 months prior to the U. S. effective filing date of this application, priority of which is claimed under 35 USC 119. [ ] YES [X] NO

COUNTRY	APP. NO.	DATE OF FOREIGN FILING

105 [ ] Specific reference to my/our related earlier filed U. S. application(s) of which this application is a [ ] CONTINUATION [ ] CONTINUATION - IN - PART [ ] DIVISION is made on page ___ of the specification for the purpose of receiving benefit of said earlier filing date(s), 35 USC 120.

400 I/We hereby appoint the following as my/our [X] attorney(s) [ ] agent(s) to prosecute this application and transact all business in the Patent Office connected therewith:

NAME	REGISTRATION NUMBER	ASSOCIATE POWER OF ATTORNEY OR AUTHORIZATION OF AGENT ATTACHED
Joseph C. Schwalbach-Louis E. Davidson	16,079 - 18,276	[X] YES [ ] NO
George R. Caruso-Robert J. Schwindaman	24,253 - 25,210	

500 SEND CORRESPONDENCE TO:

NAME	STREET	CITY AND STATE	ZIP CODE	TEL. NO.
Joseph C. Schwalbach MILES LABORATORIES, INC.,	1127 Myrtle St.,	Elkhart, IN	46514	(219) 264-8389

I/We further declare that all statements made herein of my/our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

[X] BELOW [ ] ON PAGE 2 ATTACHED

SIGNATURE(S) OF APPLICANT(S)	DATE OF SIGNATURE
Robert Charles Boguslaski	April 25, 1975
Robert Joseph Carrico	April 25, 1975
James Edward Christner	April 25, 1975

IF PAGE 2 ATTACHED, SIGN AND MAKE A PART HEREOF: [ ] YES [X] NO

IF PAGE 2 ATTACHED, SIGNING AND MAKING A PART HEREOF IS NECESSARY. ONLY PAGE 2 IS SIGNED.

(1) or made the subject of an inventor's certificate

(2) or inventor's certificate

DECLARATION AND POWER OF ATTORNEY – for use with sole or joint patent application accompanying or not accompanying the application papers

ATTORNEY'S DOCKET NUMBER (IF ANY): 11515

I/we the undersigned declare that the information (in items 201 to 206 and 301) below is true, that I/we believe that I/we am/are the original, first, and joint inventor(s) of the invention described and claimed in the specification [X] attached [ ] of my/our patent Application Serial number ____________, filed ____________, that as to common subject matter of this application and my/our earlier United States application(s), if any, described in item 105 below, I/we do not believe that the same was ever known or used in the United States before my/our invention thereof or patented or described in any printed publication in any country before my/our invention thereof or more than one year prior to such earlier application(s), or in public use or on sale in the United States more than one year prior to such earlier application(s), that the said common subject matter has not been patented(1) before the date of such earlier application(s) in any country foreign to the United States on an application, filed by me/us or my/our legal representatives or assigns more than twelve months prior to such earlier application(s) and that no application for patent(2) on said common subject matter has been filed by me/us or my/our legal representatives or assigns in any country foreign to the United States except those identified in item 600 below, if any; that, as to any subject matter of this application which is not common to any of said earlier application(s) (non-common subject matter), I/we do not know and do not believe that the same was ever known or used in the United States before my/our invention thereof or patented or described in any printed publication in any country before my/our invention thereof or more than one year prior to the date of this application, or in public use or on sale in the United States more than one year prior to the date of this application, and that said non-common subject matter has not been patented(1) in any country foreign to the United States on an application filed by me/us or my/our legal representatives or assigns more than twelve months prior to the date of this application; and that no application for patent(2) on said non-common subject matter has been filed by me/us or my/our legal representatives or assigns in any country foreign to the United States prior to this application except those identified in said item 600.

FULL NAME OF APPLICANT (INCL. AT LEAST ONE UNABBREVIATED FIRST OR MIDDLE NAME):

Item	Field	Value
201	LAST	BOGUSLASKI
	FIRST	ROBERT
	MIDDLE	CHARLES
	RESIDENCE: CITY	Elkhart
	STATE (OR FOREIGN COUNTRY)	Indiana
	COUNTRY OF CITIZENSHIP	United States
	POST OFFICE ADDRESS: STREET ADDRESS	52118 (R2) Iris Court
	CITY	Elkhart
	STATE (OR FOREIGN COUNTRY)	Indiana
	ZIPCODE	46514
202	LAST	CARRICO
	FIRST	ROBERT
	MIDDLE	JOSEPH
	RESIDENCE: CITY	Bremen
	STATE (OR FOREIGN COUNTRY)	Indiana
	COUNTRY OF CITIZENSHIP	United States
	POST OFFICE ADDRESS: STREET ADDRESS	65151 Gumwood Road R2
	CITY	Bremen
	STATE (OR FOREIGN COUNTRY)	Indiana
	ZIPCODE	46506
203	LAST	CHRISTNER
	FIRST	JAMES
	MIDDLE	EDWARD
	RESIDENCE: CITY	Ann Arbor
	STATE (OR FOREIGN COUNTRY)	Michigan
	COUNTRY OF CITIZENSHIP	United States
	POST OFFICE ADDRESS: STREET ADDRESS	1563 McIntyre
	CITY	Ann Arbor
	STATE (OR FOREIGN COUNTRY)	Michigan
	ZIPCODE	48105

LISTING OF APPLICANTS CONTINUED ON PAGE 2 HEREOF. [ ] YES [X] NO

301 TITLE OF INVENTION: SPECIFIC BINDING ASSAY METHOD AND COMPOSITION, COMPOUND, AND DEVICE FOR USE THEREIN

600 Earliest foreign application, if any, filed within 12 months prior to the U. S. effective filing date of this application, priority of which is claimed under 35 USC 119. [ ] YES [X] NO

COUNTRY	APP. NO.	DATE OF FOREIGN FILING	CONTINUED ON PAGE 2 HEREOF
			[ ] YES [ ] NO

105 [ ] Specific reference to my/our related earlier filed U. S. application(s) of which this application is a [ ] CONTINUATION [ ] CONTINUATION-IN-PART [ ] DIVISION) is made on page _____ of the specification for the purpose of receiving benefit of said earlier filing date(s) 35 USC 120.

400 I/We hereby appoint the following as my/our [X] attorney(s) [ ] agent(s) to prosecute this application and transact all business in the Patent Office connected therewith:

NAME	REGISTRATION NUMBER	ASSOCIATE POWER OF ATTORNEY OR AUTHORIZATION OF AGENT ATTACHED
Joseph C. Schwalbach-Louis E. Davidson	16,079 - 18,276	[X] YES [ ] NO
George R. Caruso-Robert J. Schwindaman	24,253 - 25,210	

500 SEND CORRESPONDENCE TO:

NAME	STREET	CITY AND STATE	ZIP CODE	TEL. NO.
Joseph C. Schwalbach, MILES LABORATORIES, INC.	1127 Myrtle St.	Elkhart, IN	46514	(219) 264-8389

I/we further declare that all statements made herein of my/our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE(S) OF APPLICANT(S) [X] HEREON [ ] ON PAGE 2 ATTACHED	DATE OF SIGNATURE
Robert Charles Boguslaski	April 25, 1975
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